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Interactions of conjugated and unconjugated bile salts with model phospholipid membranes: Vibrational spectroscopic studies

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INTERACTIONS OF CONJUGATED AND UNCONJUGATED BILE SALTS WITH MODEL PHOSPHOLIPID MEMBRANES: VIBRATIONAL SPECTROSCOPIC STUDIES

BY

Kimberly Ann Parthum
B.S. Merrimack College, 1991

DISSERTATION

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the Requirements for the Degree of

Doctor of Philosophy

in

Chemistry

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This dissertation has been examined and approved.

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7/29/96
Date

To my parents, Eric and Jane, and my sisters and "brothers," Pam, Dennis, Deb, Ray, Erica and Charlie, for their love, support and encouragement

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ABSTRACT

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Kimberly Ann Parthum
University of New Hampshire, September, 1996

Bile salts play important roles in digestion and the treatment of diseases. Few studies have been performed to determine, at the molecular level, the interactions of individual bile salt species with cellular membranes. Vibrational spectroscopy provides a noninvasive approach for characterizing the structural changes occurring within the lipid matrix due to their association with bioactive molecules. The frequency of the methylene stretching modes in the infrared spectra of the lipids comprising the membrane, as a function of temperature, yields information on the intra- and interchain order/disorder of the lipid matrix, the stability of the membrane, and the cooperativity between lipid molecules during the main phase transition. The methylene wagging modes provide information on the number and type of gauche conformers in the lipid acyl chains.

The interactions which the unconjugated and the glycine conjugated forms of sodium ursodeoxycholate (UDC) and sodium chenodeoxycholate (CDC) have on a lipid bilayer composed of dipalmitoylphosphatidylcholine, DPPC, were investigated. Experiments were performed using KH_2PO_4 hydrating solutions having initial pH's of 5, 7,

and 8. Results show that the presence of these bile salts decreases the stability of the membrane, decreases the cooperativity between the lipid molecules during the gel to liquid crystalline phase transition and increases the fluidity of the liquid crystalline phase. The degree to which the unconjugated and the glycine conjugated forms of UDC and CDC affect the DPPC bilayer is dependent on the initial pH of the hydrating solution.

Studies were also performed using hydrating solutions containing various concentrations of ethanol, which at high concentrations (0.8 to 1.2 M) causes the acyl chains in the DPPC bilayer to interdigitate. Results indicate that ethanol, at these concentrations, subdues the effects which the bile salts have on the lipid bilayer.

The methylene wagging modes provided information on the effects which the bile salts and ethanol have on the number and type of gauche conformers in the acyl chains. The results obtained for the methylene wagging and symmetric stretching regions were compared. Some differences were noted for the lipid bilayer in the gel phase, while, the results for the liquid crystalline phase were similar.

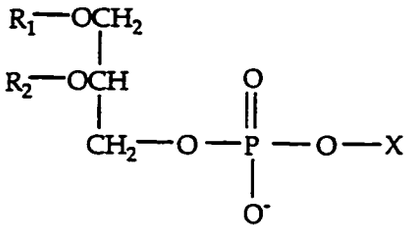
CHAPTER 1

INTRODUCTION

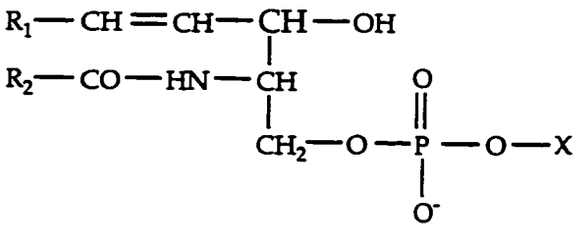
1.1 Lipids

Singer and Nicolson, in 1972, proposed a model for the structure of biological membranes called the fluid mosaic model. This model describes the membrane as a "sea" of lipid molecules in which protein "icebergs" float.[1, 2] There are three types of lipids in cell membranes. They are: 1) phospholipids, 2) sphingolipids, and 3) glycolipids.[3] A schematic representation of the structures of these lipids is given in Figure 1.1. The lipids are derived from several compounds. For example, the phospholipids are derivatives of glycerol and the sphingolipids are derivatives of sphingosine. The acyl chains of the lipids consist of saturated or unsaturated hydrocarbon chains that contain typically between 10 and 28 carbons. Various headgroups are attached to the acyl chains. Among the most common headgroups are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol. The structures for these headgroups are given in Figure 1.2.

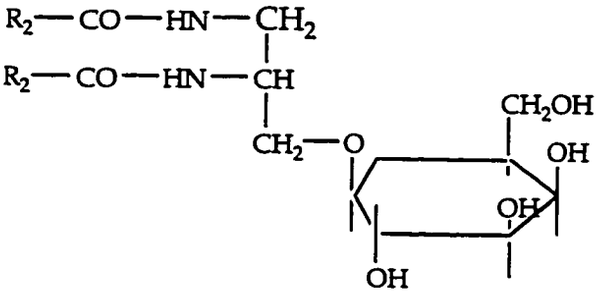
The composition and distribution of lipids vary widely in membranes. The most abundant lipids in animal cell membranes are the phospholipids.[2] Phospholipids are approximately 40-90% of the total dry weight of most membranes.[4] The phospholipids are formed from the esterification of two fatty acids.[2] Phospholipids differ in the number of carbons in their acyl chains and in the nature of their headgroup. Glycerophospholipids are the major class of naturally occurring phospholipids and are



Phospholipid

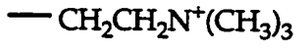


Sphingolipid

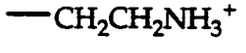


Glycolipid

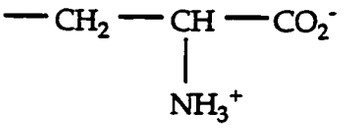
Figure 1.1: Molecular Structures of Various Lipids in Cell Membranes
X = Headgroup; R_1, R_2 = Hydrocarbon Chain.



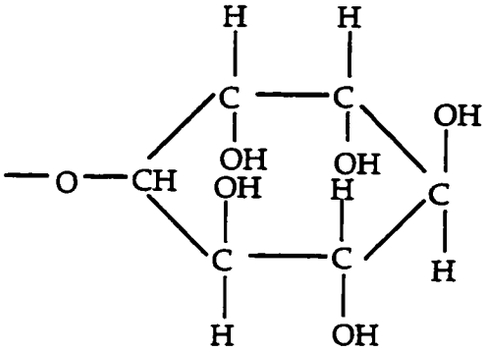
Phosphatidylcholine



Phosphatidylethanolamine



Phosphatidylserine



Phosphatidylinositol

Figure 1.2: Molecular Structures of Common Headgroups (X)

derivatives of glycerol-3-phosphates. They consist of a very polar headgroup, and nonpolar, hydrophobic, acyl chains. The phosphatidylcholines are phosphorylated derivatives of glycerol with a phosphatidylcholine headgroup.[3] The most widely studied phospholipid is dipalmitoylphosphatidylcholine, DPPC. The structure of DPPC is given in Figure 1.3.

Phospholipids spontaneously assemble into bilayers, when hydrated with an aqueous solution. These bilayers have the polar headgroup facing the aqueous medium and the nonpolar acyl chains facing inward away from the aqueous medium. Liposomes are vesicles in which there is an aqueous region enclosed by a layer of lipid molecules.[3] Vesicles can range in size from nanometers to microns. Liposomes are prepared using procedures that control the size of the vesicle and the number of bilayers. There are four types of liposomes; 1.) small unilamellar vesicles (SUVs), 2.) intermediate-sized unilamellar vesicles (IUVs), 3.) large unilamellar vesicles (LUVs), and 4.) multilamellar vesicles (MLVs). Schematic representations of these liposomes are given in Figure 1.4. The SUVs are typically 200 Å to 500 Å in diameter.[3, 5] SUVs can be produced by ultrasonification of MLVs. Ultrasonification causes the MLVs to breakdown, forming SUVs. The MLVs and the LUVs are the largest sized vesicles, typically 500 Å to 5000 Å in diameter.[3, 5] LUVs are produced by adding an excess of a detergent to the lipid solution and then removing the detergent by diluting with an organic solvent. Another method used to form LUVs involves the extrusion of a lipid dispersion of MLVs through a filter of known pore size.[5, 6] MLVs are composed of at least five concentric lamellae with a layer of water separating each individual lamella. MLVs are typically prepared by

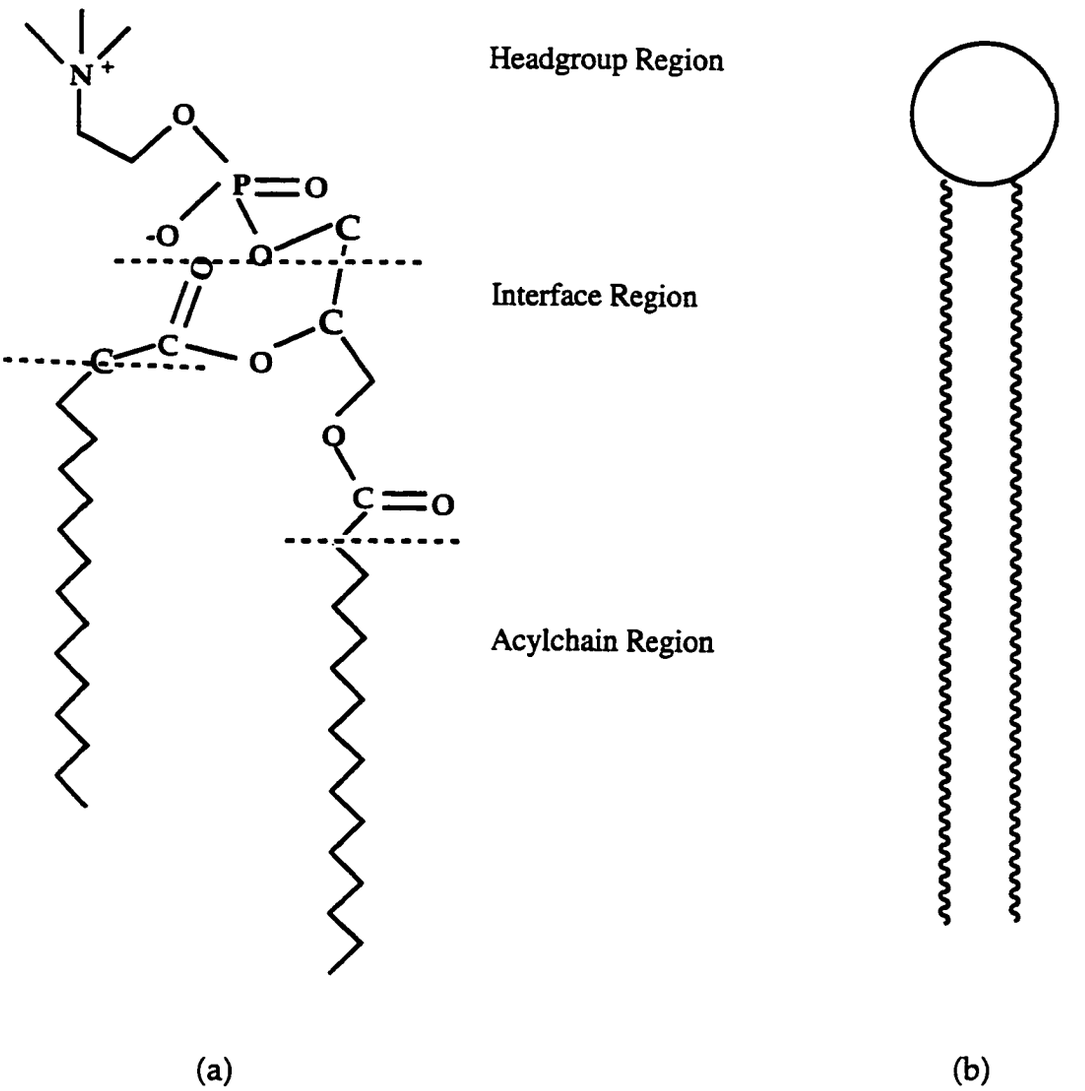
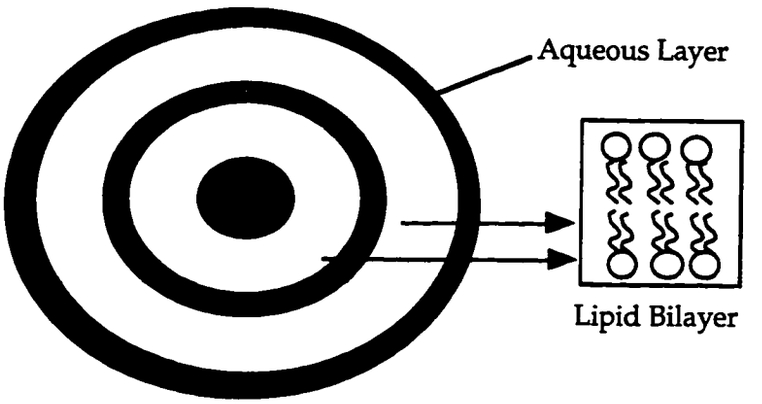
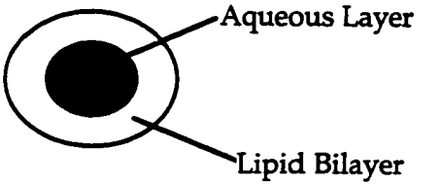


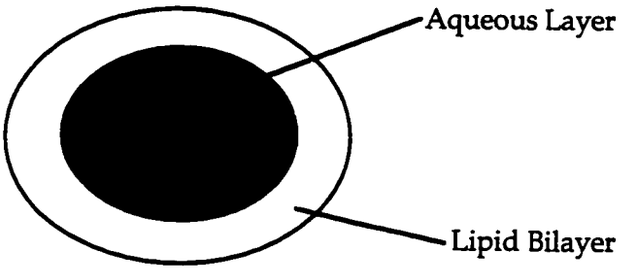
Figure 1.3: a) Molecular Structure of Dipalmitoylphosphatidylcholine (DPPC)
b) Schematic Representation of DPPC



Multilamellar Vessicle (MLV)



Small Unilamellar Vesicle (SUV, 200-500Å)



Large Unilamellar Vesicles (LUV, 500-5000Å)

Figure 1.4: Schematic Representation of the Types of Liposomes

hydrating the lipid with an excess of the aqueous hydrating solution.

Many studies have been performed using SUVs, LUVs, and MLVs to determine the size and lipid composition of the vesicles and to evaluate the effects which the addition of certain species has on the vesicle.[6-12] Mayer et al. conducted experiments to investigate the effect of combining freeze-thaw and extrusion techniques to produce different sized vesicles using filters of various pore sizes.[6] They used ^{31}P-NMR to determine if MLVs or LUVs of egg phosphatidylcholine were formed. Results indicated that when pore sizes of 40 and 200 nm were used, the extruded vesicles formed were mainly MLVs. When vesicles were formed by a combination of the freeze-thaw and extrusion methods, a higher percentage of the formed vesicles were LUVs. Their studies showed that when the freeze-thaw method and a 200 nm pore sized filter was utilized 75 percent were LUVs whereas when only the extrusion technique was performed, about 20 percent were LUVs. Their results also indicated that the vesicles formed are all similar in size and the size of the vesicle formed depends on the pore size of the filter.[6]

Benachir and Lafleur studied the effect of melittin, a peptide found in bee venom, on LUVs composed of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC).[12] They monitored the release of calcein, a fluorescent marker, from LUVs through the use of fluorescence spectroscopy. Results showed that the addition of melittin to the vesicles caused the release of the calcein. The release of calcein was found to follow an all-or-none pathway. That is, two types of vesicles exist: 1.) intact vesicles with a calcein concentration after the addition of melittin equal to the initial concentration of calcein, and 2.) empty vesicles which have totally released the calcein. It was suggested that there are

250 melittin molecules per vesicle needed to induce complete release of calcein from the vesicle. If the number of melittin molecules per vesicle is lower than 250, no leakage will occur. The number of melittin molecules needed to induce the release of calcein is high which suggests the leakage may be due to the melittin perturbing the bilayer rather than forming pores within the bilayer. These studies led to an increase in the knowledge of the mechanism of how the molecules permeate through the bilayer and how the molecules affect the bilayer.[12]

DPPC, with the addition of water, spontaneously forms bilayers which are arranged in multilamellar vesicles. A schematic representation of the lipid bilayer is shown in Figure 1.5. The bilayer isolates cell and organelle contents from the external environment.[2] The bilayer also provides a supporting matrix for the proteins involved in transport across the bilayer, signal transduction, and metabolism.[2] The fluidity of the bilayer determines the degree of structural stability. If there is excessive conformational disorder, the bilayer is destabilized and collapses or loses its selective permeability.[2] Bilayer fluidity also regulates the activity of many proteins, such as, conformational transitions. If the bilayer is too rigid, these transitions can be restricted or prevented.

The bilayer may exist in the more ordered, rigid, gel state, at temperatures below the phase transition temperature, or the more disordered, liquid crystalline state.[13] When the bilayer transforms from the gel state to the liquid crystalline state, the acyl chains are said to "melt." When the acyl chains "melt," gauche conformers are introduced. In the case of DPPC, approximately five gauche conformers per a chain are introduced during the gel to liquid crystalline phase transition.[14, 15] The bilayer thickness in the gel

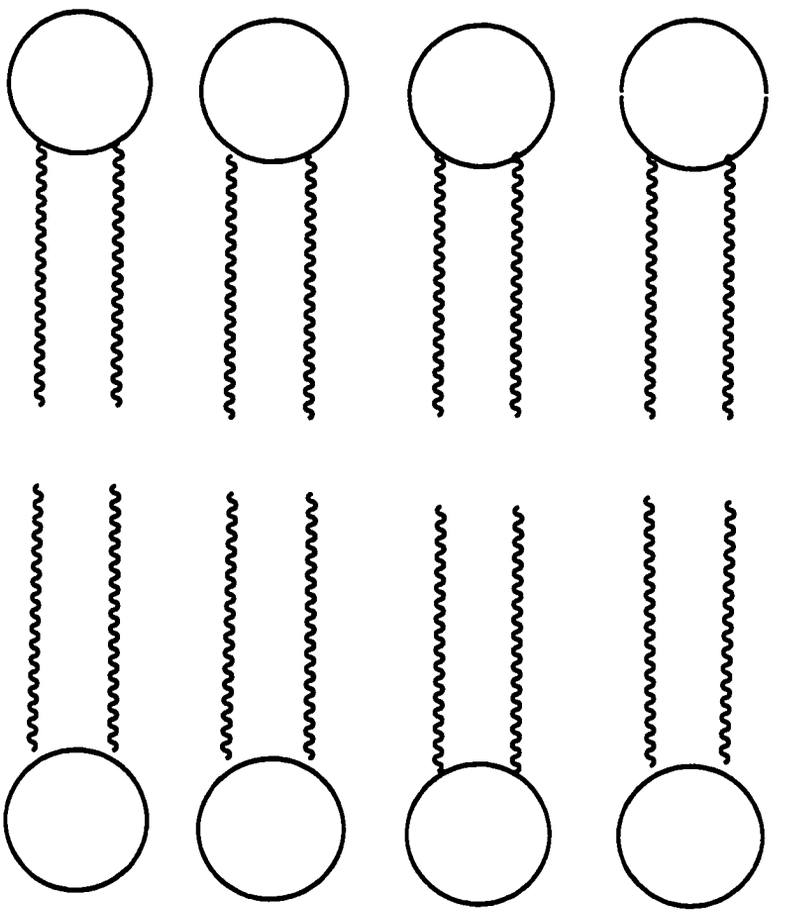


Figure 1.5: Schematic Representation of the Lipid Bilayer

phase is approximately 42 Å while in the liquid crystalline phase the bilayer thickness drops to about 35.5 Å.[1] This shift in thickness is believed to be due to the introduction of gauche conformers.[1] The gel to liquid crystalline phase transition is called the main phase transition and it occurs at a temperature between 40 °C and 42 °C for multilamellar vesicles composed of DPPC. There are two other transitions that occur for DPPC, the subtransition and the pretransition. The subtransition occurs at approximately 18 °C after the sample has been incubated at 0 °C for a period of time. The pretransition occurs at approximately 35 °C. The subtransition results in a change in the packing pattern of the lipid's acyl chains while the pretransition induces slight changes, as compared to the changes observed in the bilayer during the main phase transition, in the bilayer structure.[2, 4, 14]

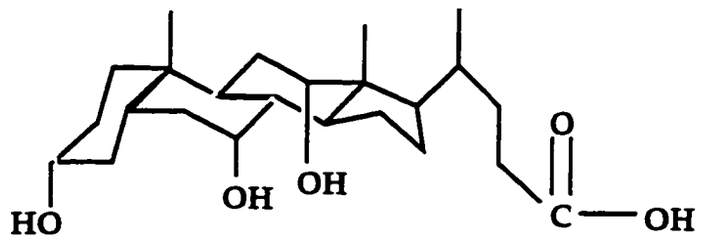
The cooperativity of the lipid molecules during the phase transition describes the behavior of the lipid's acyl chains (ie., do the acyl chains "kink" simultaneously or in a more random fashion due to the introduction of gauche conformers). Cooperativity of the lipid molecules during the phase transition affects the temperature range over which the phase transition occurs. The more narrow the temperature range, the more cooperative the phase transition. Similarly the wider the temperature range of the "melt," the less cooperative the phase transition. The order parameter defines the degree of angular disorder of the molecules.[3] Basically, the order parameter describes how the hydrocarbon chains are aligned in the bilayer. It can be used to describe the fluidity of the bilayer. If the order of the bilayer decreases, the bilayer becomes more fluid, whereas an increase in the order indicates a less fluid bilayer. The order of the hydrocarbon chains is

dependent on temperature and the components of the lipid assembly.[1] These parameters, cooperativity between the lipid molecules in the bilayer during the phase transition, order of the phase(s) and phase transition temperature, provide information on the effects which species (ie., bile salts, ethanol, metal ions, etc.) have on the lipid assembly.

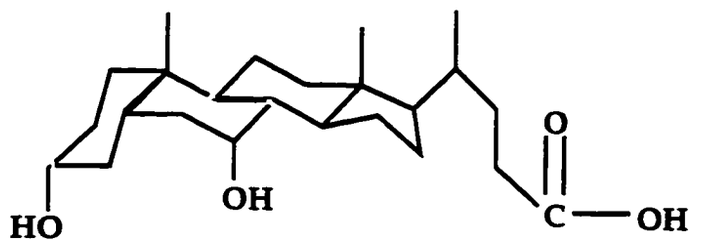
1.2 Bile Salts

The bile salts are rigid, naturally occurring surfactants capable of forming micelles. They have many isomeric, epimeric and conjugated forms. The bile salts play important roles in digestion and in the treatment of diseases.[16-29] Currently, the unconjugated form of ursodeoxycholate (UDC) is used in the United Stated to dissolve gallstones.[20-28] The common bile salts differ in the orientation and number of hydroxyl groups. The orientation of the hydroxyl group allows the bile salts to have both hydrophilic and lipophilic surfaces. The bile salts are products of cholesterol metabolism and are the major components in human bile.[3]

Human bile is composed of phospholipids, cholesterol, proteins, bilirubin, electrolytes and bile salts. Bile salts are the major component (67%) of human bile.[17] There are two primary bile salts which exist in the bile of humans, cholic and chenodeoxycholic acid. These two bile salts are converted in the colon to the secondary bile acids, deoxycholic and lithocholic acid, respectively. The molecular structures of the primary and secondary bile salts are given in Figure 1.6. The primary bile salts represent 70 percent of the total bile salt composition in human bile, whereas the secondary bile



(a)



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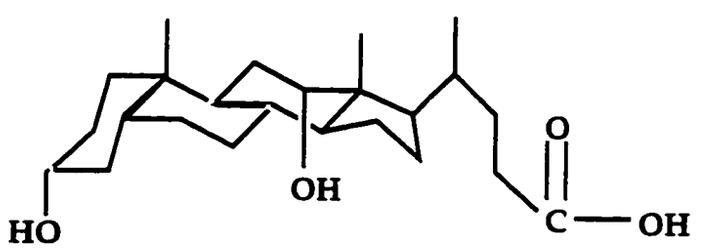

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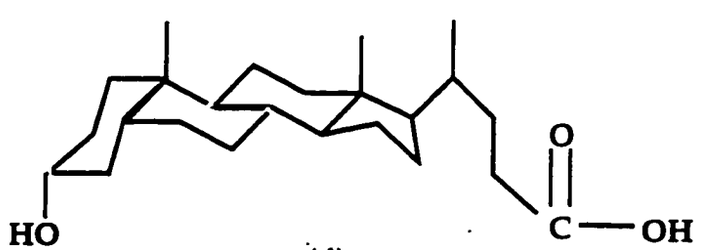


(d)

Figure 1.6: Molecular Structures of the Primary Bile Acids, a) Cholic Acid and b) Chenodeoxycholic Acid, and the Secondary Bile Acids c) Deoxycholic Acid and d) Lithocholic Acid

salts, deoxycholate and lithocholate, represent 25 and 1 percent, respectively.[16] The small percentage of lithocholate present in the body is due to it being poorly absorbed in the intestine.[18] Lithocholate is either amidated, at the acid functionality, or sulfated thru the hydroxyl group at the three position. Several other bile salts, including ursodeoxycholate, comprise the remaining percentage of bile salts in human bile.[16]

The bile salts are conjugated in the liver with either taurine or glycine. The majority of bile salts in the body, 75 percent, exist as the glycine conjugates.[16] A smaller percentage, 24.8 percent, exists as the taurine conjugates. The percentage of bile salts existing in the unconjugated form is only about 0.2 percent.[16] During digestion, the gallbladder contracts and the bile salts are secreted, in bile, into the small intestine where they aid in the digestion of fats.[18] The majority of the bile salts are reabsorbed in the terminal ileum and returned to the liver.[18] In the liver they are reconjugated, if necessary, resecreted into the bile and stored in the gallbladder until they are required again for digestion.[16, 18] This process is known as enterohepatic circulation and conserves the quantity of bile acids which exist in the body. The entire bile salt pool (3.5 g) recirculates 8 to 10 times a day (approximately twice with each meal).[16, 17, 29]

Bile salts self aggregate and form micelles in aqueous solution. Typically, the hydrophilic face is exposed to the aqueous environment while the hydrophobic face is away from the aqueous environment, thus forming a normal micelle.[16] If the bile salt is in a less hydrophilic environment, the opposite structure is formed. That is, the hydrophilic face is away from the less polar environment and the hydrophobic face is toward the nonpolar environment.[16] This type of micelle is known as a "reversed

micelle." Self aggregation occurs when a certain concentration, the critical micellar concentration (cmc), is reached. Primary micelles are formed at concentrations just above the cmc and consist of 2-13 molecules.[16] At higher concentrations of bile salt, the primary micelles aggregate and form secondary micelles.[16] Two types of bile salt micelles can exist. They are simple micelles, composed of only bile salt molecules, and mixed micelles, composed of bile salts and other species. Typically, the other species incorporated into the micelles are lipids and/or cholesterol.

The same properties which allow the bile salts to solubilize fats also allows them to interact with the more hydrophobic lipids in the membranes causing the membrane structure to be altered.[30, 31] The interactions which bile salts have with membranes can occur in several ways.[16] They may bind or dissolve within the lipid bilayer.[16] Bile salts increase the fluidity of the membrane by binding at low bile salt concentrations.[16] Bile salts can also "cross membranes" by dissolving into the lipid bilayer and form channels which allow other species (such as, ions or organic molecules) to enter the bilayer.[16] At high bile salt concentrations, bile salts can solubilize the phospholipids and cholesterol in the membrane and can cause the release of proteins.[16] This may lead to severe damage to the membrane which could interfere with the functions of the membrane. Many studies have been performed, at high mole ratios of bile salt to lipid, to gain an understanding of the solubilization of lipids by bile salt micelles.[32-39] Few studies, however, have been performed to determine, at the molecular level, the interactions of individual bile salt species with cellular membranes.[40-42]

1.3 Instrumental Methods Used to Study Membranes

Many techniques have been used to obtain information about the motion of the different regions of the bilayer and changes in the structure of lipid bilayers. Experiments have been performed using fluorescence and electron paramagnetic resonance spectroscopies.[12, 43-45] These two techniques require that a molecular probe be inserted into the bilayer to obtain information on the lipid bilayer. Some studies monitored the release of certain compounds from the lipid bilayer.[12, 43] Techniques which do not require the use of a probe include differential scanning calorimetry (DSC), ^{31}P-, ^{2}H-, ^{1}H-, and ^{13}C-nuclear magnetic resonance (NMR), Raman and infrared spectroscopies.

McElhaney has reviewed the use of DSC for studying the thermotropic behavior of lipids in real and model membranes.[46] DSC can be used to determine the phase transition temperature (T_m), enthalpy of the phase transition and the cooperativity between the lipid molecules during the phase transition. DSC has been used to study pure phospholipids, phospholipids having different acyl chains, and binary mixtures of phospholipids.[46] Both SUVs and MLVs have been studied. The effects which pH has on the phase transition of specific lipid bilayers has been monitored.[46] The addition of various species to lipid bilayers also has been examined.[46]

Many studies have been performed using various types of NMR spectroscopy.[47-54] Information regarding the headgroup has been obtained by ^{31}P-NMR. This type of NMR spectroscopy monitors the mobility of the headgroup, and perturbations to the headgroup due to species added to the lipid bilayer.[47-54] The shape of the band in the ^{31}P-NMR spectrum can be used to identify the state (ie., bilayer, hexagonal or micellar) in

which the lipid exists.[47-50, 52] ^{2}H-NMR experiments usually require certain regions of the acyl chain to be deuterated to obtain molecular order information.[49, 50, 52] Molecular order information can also be obtained through the use of either ^{1}H- or ^{13}C-NMR.[53, 54] These two techniques can also provide information concerning the motion of the different regions in the lipid bilayer.

The studies performed here focus on the use of vibrational spectroscopy to study model membranes. Infrared and Raman spectroscopy provide a noninvasive means for determining the conformational and dynamical properties of the lipid bilayer.[1] Information on how the different sections of the lipid molecule in the membrane are affected by the presence of an added species can be determined by monitoring different regions of the spectrum. For example, spectral changes due to the lipid's carbon skeleton are indicative of intramolecular changes in the lipid's acyl chains, while spectral changes due to the terminal methyl group of the lipid's acyl chains are indicative of intermolecular interactions between the lipid's acyl chains.

Many studies have been performed using Raman spectroscopy to determine the effects which various species have on membranes.[55-60] The gel to liquid crystalline phase transition involves intrachain trans/gauche isomerization and expansion of the hydrocarbon chain lattice.[4, 61] Changes in the intermolecular order chain packing characteristics are seen in changes in the intensity and the frequency of the carbon hydrogen stretching modes.[1, 61] The hydrocarbon stretching region is useful in determining the behavior of either highly or moderately cooperative gel to liquid crystalline phase transitions.[4, 62, 63] The useful C-H stretching peaks in Raman

spectroscopy are observed at 2850, 2880 and 2935 cm^{-1}. Typical Raman spectra for pure DPPC hydrated with a 50 mM KH_2PO_4 solution, initial pH of 5, containing 20 mg/ml ethanol-d_6 are given in Figures 1.7 and 1.8. One usually observes a decrease in the intensity of the 2880 cm^{-1} peak and a shift of 10-12 cm^{-1}, to higher wavenumbers, in the frequency of this peak.[1, 4] Generally, the ratio of the intensity of the 2935 cm^{-1} band to the intensity of the 2880 cm^{-1} band is used to monitor primarily intermolecular chain-chain disorder effects for the lipid's acyl chains with changes in conformation of the lipid acyl chains (ie., increase in the number of gauche conformers), superimposed to some degree. The ratio of the intensity of the 2850 cm^{-1} band to the intensity of the 2880 cm^{-1} band provides more direct information with respect to the lateral chain-chain effects reflecting intermolecular chain-chain disorder.[1, 4, 63]

The effects which various species (ie., anesthetics, tumor promoters, vitamins, proteins, etc.) have on the lipid assembly have been monitored using Raman spectroscopy. Craig et al. determined the effects of halothane, an anesthetic, on dipalmitoylphosphatidylcholine (DPPC) liposomes using Raman spectroscopy.[56] They monitored changes in the peak height intensity ratios of the 2935 cm^{-1} band to the 2880 cm^{-1} band and of the 1090 cm^{-1} band to the 1130 cm^{-1} band. These ratios correspond to bands in the C-H stretching region and the C-C stretching region, respectively. Results showed that halothane reduced the cooperativity of the phase transition and destabilized the DPPC bilayer. There did not appear to be a significant change in the order parameters for either the gel or the liquid crystalline phase. The authors concluded that the halothane is interacting with the headgroup of the lipid bilayer.[56]

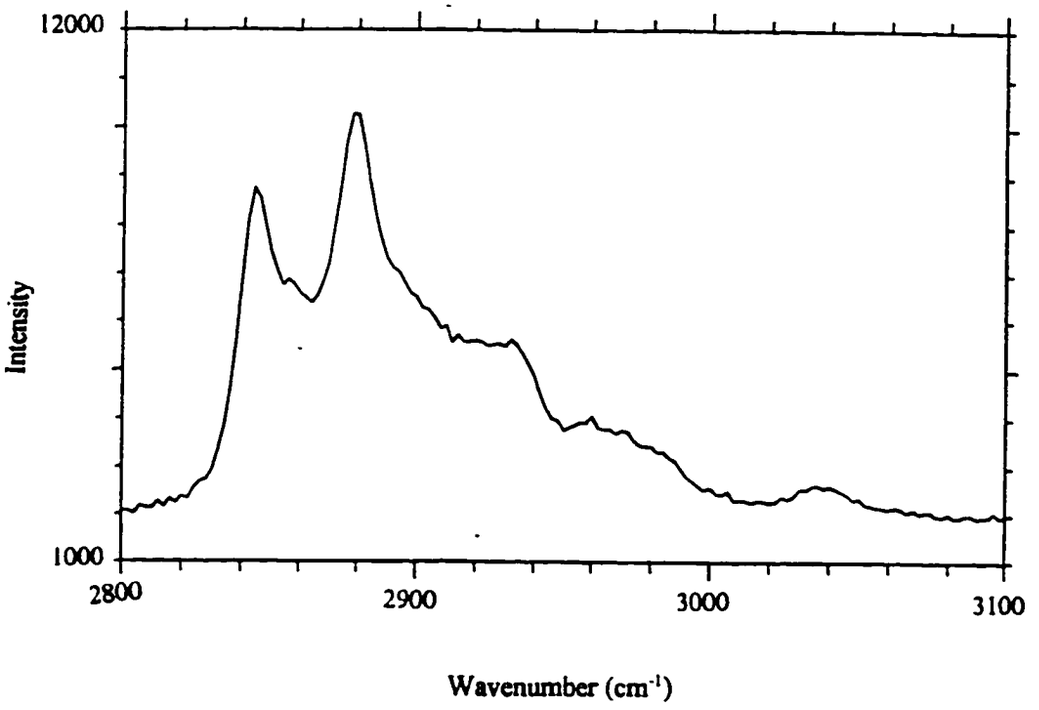


Figure 1.7: Raman Spectrum of Pure DPPC Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5, 32 °C)

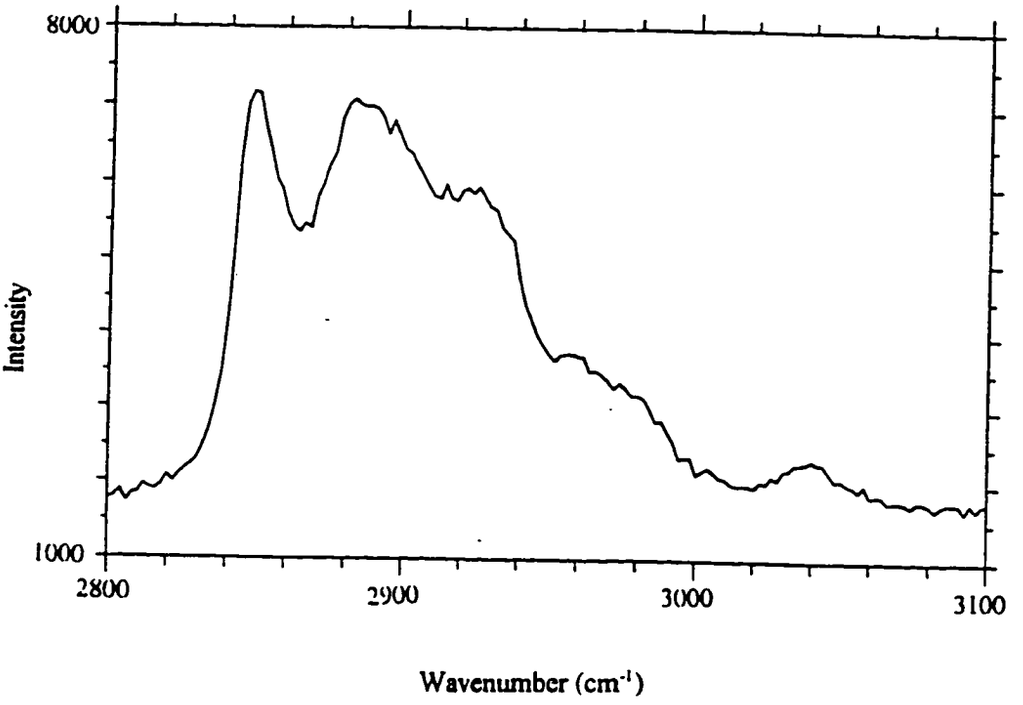


Figure 1.8: Raman Spectrum of Pure DPPC Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5, 44 °C)

Lafleur et al. studied the effect which melittin, a peptide in bee venom, has on the thermotropic properties of DPPC using Raman spectroscopy.[64] They monitored changes in the intensities of bands in the C-H and C-C stretching regions. The changes which were observed in the C-C region were not as dramatic as those observed in the C-H region. The C-H stretching region was affected more by the addition of melittin. Results showed that the cooperativity between the lipid molecules during the gel to liquid crystalline phase transition decreased for the melittin containing samples when compared to pure DPPC. The stability of the bilayer also decreased with the addition of melittin. Changes were also observed in the level of acyl chain disorder for the melittin containing samples. The level of acyl chain disorder in the liquid crystalline phase increased.[64]

Application of infrared spectroscopy to the analysis of membrane structure was pioneered in the 1960's.[11, 14] Infrared spectroscopy is also an excellent method for the study of membrane systems.[65-68] This is due to the fact that: 1.) both structural and dynamic data can be obtained from the molecules being studied, without the use of probes, 2.) information from several regions of the infrared spectrum can be obtained from a single experiment, and 3.) sample sizes can be small.[13] However, there are many difficulties associated with using infrared spectroscopy to analyze samples hydrated with aqueous solutions. One problem is that water absorbs strongly in the infrared region of the spectrum. The use of short pathlengths, deuterated water (D_2O), or attenuated total reflectance spectroscopy helps to minimize this problem.[13]

A typical infrared spectrum of a multilamellar assembly composed of DPPC is given in Figure 1.9. The methyl and methylene stretching bands, 3100-2800 cm^{-1}, are the

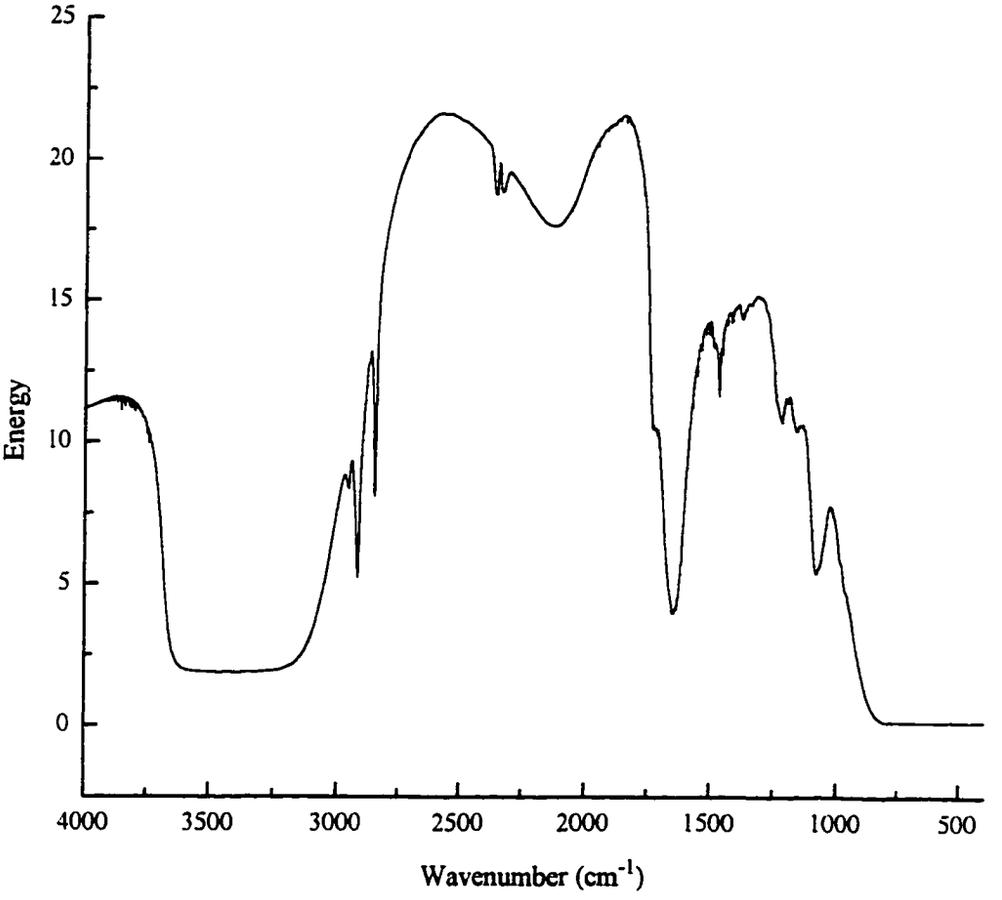


Figure 1.9: Infrared Spectrum of Pure DPPC Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7, 32 °C)

most intense bands in the spectrum.[13, 14] The methylene stretching bands are generally monitored and are sensitive to changes in the conformation (ie., trans/gauche ratio) of the lipid's acyl chains.[13, 14] Other modes that are used to determine information about the acyl chains are the bending vibrations for the methylene and methyl groups in the region of 1500-1350 cm^{-1}, the methylene wag between 1380-1180 cm^{-1}, and the methylene rocking bands in the region of 1150-700 cm^{-1}. The methylene wag and the methylene rocking bands are difficult to utilize.[13, 14] The methylene wag overlaps the strong asymmetric stretching band of the phosphate group and the rocking band overlaps a strong water band.[13, 14] Studies have been performed, however, which monitor the intensity of the weak CH_2 wagging mode.[69-72] The interfacial region is monitored using the carbonyl stretching modes in the region of 1750-1700 cm^{-1}. These bands are the most useful to monitor changes occurring in the interfacial region since the C-O stretching band couples with the C-C stretching vibration.[13, 14] The head group region can be monitored by using the C-N stretching bands (1040-800 cm^{-1}) or the P=O stretching modes (1228-1100 cm^{-1}). The P-O stretching band is located within the range of 900 - 800 cm^{-1} is obscured, however, by water bands.[13] Our studies have concentrated primarily on monitoring the methylene stretching bands due to their intensity and sensitivity to conformational changes. The methylene wagging modes were also studied to confirm that changes observed in the frequency of the methylene symmetric stretching band were due primarily to conformational changes and not due to other contributing factors.

A thermotropic phase transition profile for DPPC is obtained by plotting the methylene symmetric stretching band frequency versus temperature. An example of a

typical phase transition profile for pure DPPC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 is given in Figure 1.10. The methylene symmetric stretching mode at 2850 cm^{-1} is generally used for this purpose. The methylene asymmetric stretching mode at 2920 cm^{-1} undergoes larger changes, however it is affected more by spectral overlap with the broad, underlying, water absorption band.[14] The phase transition profile provides information on the cooperativity between lipid molecules during the phase transition, the fluidity of both the gel and the liquid crystalline phases, and the stability of the bilayer. The following information is used to aid in the interpretation of the phase transition profiles:

1. A shift in band position to a higher wavenumber indicates that the chains have more gauche conformers (or kinking) thus resulting in a more "disordered" phase. A bilayer which is more disordered is also more fluid. Conversely, a shift in band position to lower wavenumbers indicates that the lipid's acyl chains have more trans conformers and are more ordered. A more ordered bilayer is less fluid.

2. A sharp phase transition profile indicates there is a high degree of cooperativity between the lipid molecules during the phase transition whereas a broad phase transition profile indicates a lower degree of cooperativity between the lipid molecules during the phase transition.

3. A shift in the phase transition temperature to lower temperatures indicates that the membrane has been destabilized.

Comparing the phase transition profiles obtained for pure DPPC with the phase transition profiles of DPPC samples containing an added species, allows for the determination of the

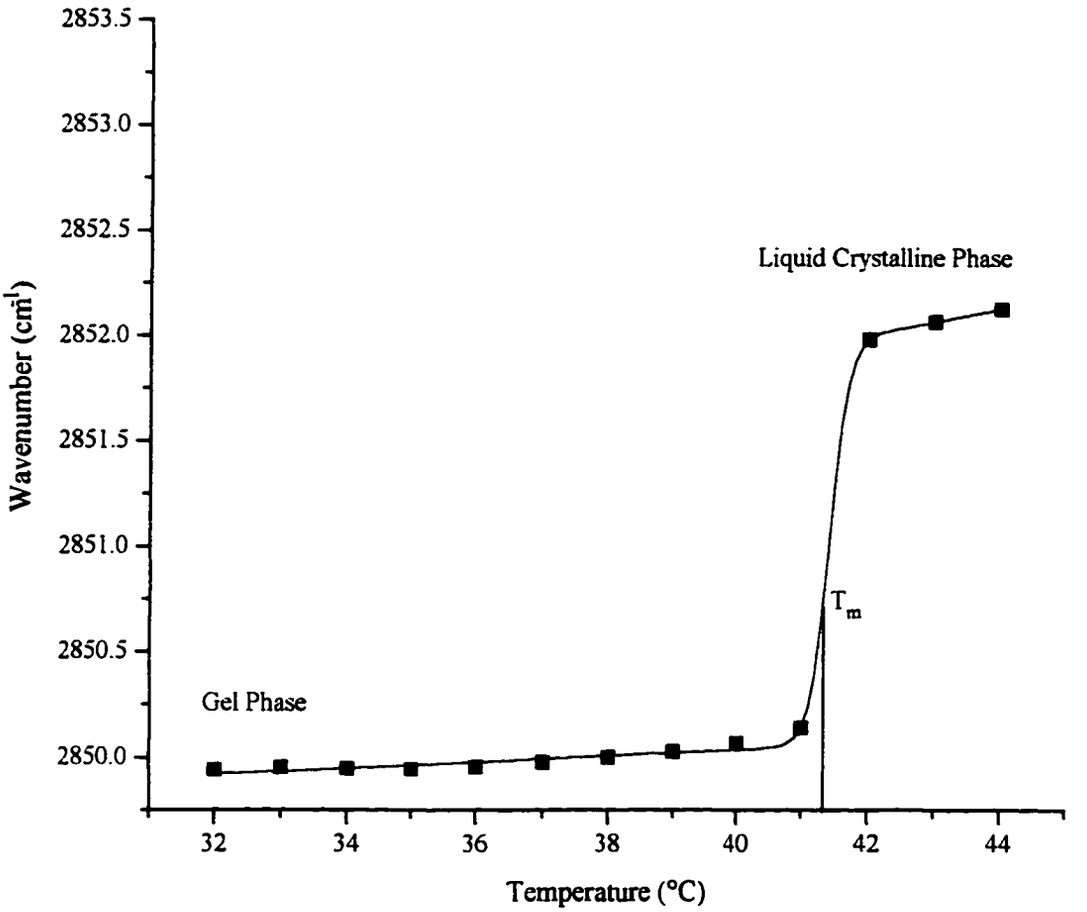


Figure 1.10: Phase Transition Profile of Pure DPPC Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7)

effects, if any, which the addition of a species (ie., bile salt, ethanol, etc.) has on the lipid dispersion.

Many studies have been performed using infrared spectroscopy to determine the interactions of various species with the lipid bilayer, and to determine how the species interact with the lipids. Ortiz et al. monitored the interaction of vitamin K_1 with phospholipid vesicles by infrared spectroscopy.[73] They used multilamellar liposomes composed of DPPC. Changes in the frequencies of the bands in the methylene stretching region were monitored. The gel to liquid crystalline phase transition temperature of the DPPC bilayer was shown to decrease with increasing concentrations of K_1. The frequency of the asymmetric methylene stretching band did not change in the gel or the liquid crystalline phase when compared to pure DPPC. However, when the half-height bandwidths of the asymmetric stretching band (obtained from the spectra) were plotted versus temperature a decrease in the bandwidth was observed in the liquid crystalline phase for the K_1 containing sample. The authors concluded that this decrease in bandwidth indicates a decrease in librational and torsional motion for the liquid crystalline phase. No change in motion was observed for the gel phase.

Nam et al. determined the distribution of tumor promoters, 12-O-tetradecanylphorbol-13-acetate (TPA) and teleocidin, in lipid membranes using Fourier transform infrared (FTIR) spectroscopy.[43] Experiments were performed with multilamellar vesicles of DPPC. Changes in the frequencies of the methylene stretching bands were monitored. The results of their experiments showed that the frequency of the methylene symmetric stretching band was not affected significantly by the addition of these

tumor promoters to the lipid bilayer. The bandwidth , however, did show changes with the addition of TPA, a tumor promoter possessing biological activity. The bandwidth was shown to decrease with the addition of TPA. The authors attribute the decrease in bandwidth to a restriction in the librational and torsional motions of the lipid molecules in the bilayer. They also monitored changes in the position of the C=O band of the lipid. No changes were observed in the bandwidth for this band which led the authors to suggest that the TPA was mainly interacting with the hydrophobic core of the bilayer. The effect which teleocidin, another tumor promoter with biological activity, has on the bilayer was also monitored. Changes in the bandwidth for the C-H stretching band and the C=O stretching band were observed for the samples containing teleocidin. Since the bandwidths of both peaks are affected, the authors conclude that teleocidin is located on the surface of the membrane.

1.4 Goal of the Research

The purpose of the research described here, was to investigate the effects which several bile salt species have on a model membrane system composed of multilamellar vesicles of DPPC using vibrational spectroscopy, a noninvasive technique, and to gain an understanding of the interaction which the bile salts have with model membranes. These studies are important because bile salts are present in the human body and play a significant role in digestion. Many studies have been performed to determine the physical properties of bile salts, but few studies have been performed to determine the nature of the bile salt/lipid interactions on a molecular level. Bile salts can have protective or toxic

properties towards cell membranes depending on their molecular structure. The effects which the bile salts have on cell membranes is due to a variety of factors. One factor is the hydrophobicity of the bile salt. It has been determined that the more hydrophobic bile salts are more toxic.[30] It is has also been shown that the bile salts have different properties in solutions of different pH. Protonated bile salts are more lipophilic which indicates the protonated bile salts are more likely to partition into the lipid membrane.[28] It is possible that the protonated and the unprotonated forms of the bile salts interact with the lipid assembly differently. The effect which the addition of ethanol to the hydrating solution has on the bile salt/DPPC interactions is also important to study. This is due to the fact that ethanol and bile salts are both present in the small intestine during the consumption of alcoholic substances and ethanol, at high concentrations, is known to cause interdigitation of the DPPC bilayer and cellular membranes. Studies which monitor the interactions of different bile salts with model membranes hydrated under various conditions were performed.

Specifically, the effects which the two bile salt epimers, sodium ursodeoxycholate and sodium chenodeoxycholate, have on the DPPC bilayer were studied. Studies were performed using both the unconjugated and the glycine conjugated forms of these bile salts. The interactions of these bile salts with the lipid bilayer were studied under various hydrating conditions to determine the effects which: 1.) the pH of the hydrating solution, 2.) the presence of ethanol, and 3.) the concentration of the counterions, have on the interactions which the unconjugated and glycine conjugated bile salts have with the DPPC bilayer.

CHAPTER 2

EVALUATION OF THE INTERACTIONS OF THE UNCONJUGATED BILE SALTS, SODIUM URSODEOXYCHOLATE AND SODIUM CHENODEOXYCHOLATE, WITH DPPC MULTILAMELLAR VESICLES

2.1 Introduction

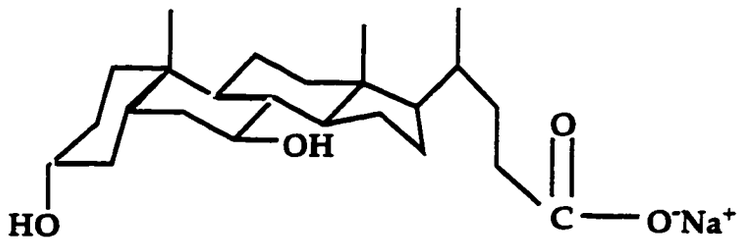
Bile salts in their many isomeric, epimeric and conjugated forms play important roles in digestion and the treatment of diseases. Bile salts form micelles which aid in the solubilization of fatty acids, phospholipids and cholesterol. Many studies have been performed at high mole ratios of bile salt to lipid to gain an understanding of the solubilization of lipids by bile salt micelles, including the size and shape of the mixed micelles formed.[32-39] The bile salts exist in the body mainly as the taurine and the glycine conjugates. A small percentage of the bile salts in the body do exist in the unconjugated form. Since several of the unconjugated bile salts have been used for medicinal purposes, an understanding of the effects of unconjugated bile salts on lipid assemblies is important.

In the present study infrared spectroscopy was utilized to examine the effects of bile salt species on multilamellar assemblies prepared from the saturated chain lipid, dipalmitoylphosphatidylcholine (DPPC). Specifically, the effects of two bile salts, sodium ursodeoxycholate (UDC) and sodium chenodeoxycholate (CDC), on the acyl chain properties of DPPC bilayers were investigated. The molecular structures of these bile

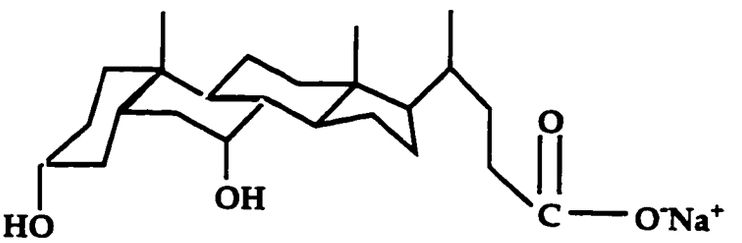
salts are given in Figure 2.1. These two dihydroxy bile salts are epimers, but are known to interact differently with cell membranes. It is known that CDC disrupts cell membranes, while UDC has been observed to exhibit cytoprotective properties for hepatocytes.[30, 41, 44, 74-76] UDC has also been used for many years as a "folk drug" in Asian medicine [19] and is currently employed as an effective therapeutic agent for dissolving gallstones and in treating numerous liver diseases.[20-28] The side effects associated with the use of CDC have limited its use in the United States.[77-80]

Studies have shown that the hydrophobic bile salts are more toxic than the hydrophilic bile salts.[30] The hydrophobicities of the bile salts can be determined through the use of high performance liquid chromatography (HPLC). Results from experiments which utilized HPLC on the bile salt monomers indicate that the CDC epimer is more hydrophobic than the UDC epimer.[81-83] The differences in hydrophobicities of these two epimers is due to the orientation of the 7-hydroxyl group.[30, 41, 84] CDC has the 7-hydroxyl group located in an axial position, while for the UDC epimer, this group is equatorial.[85] In addition to differences in orientation of the 7-hydroxyl group between CDC and UDC, the two bile salts differ in their rates of conversion of each bile salt, in the human body, to lithocholic acid, a more toxic bile acid. The conversion of CDC to lithocholic acid, in the intestines, occurs more rapidly and is more complete than for the UDC epimer, which may provide additional insight into the cytotoxic properties of CDC.[79, 86]

Heuman et al. have studied the protective effects of UDC on large unilamellar vesicles composed of egg phosphatidylcholine.[41] The release of tritiated inulin was



(a)



(b)

Figure 2.1: Molecular Structures of a) Sodium Ursodeoxycholate and b) Sodium Chenodeoxycholate

monitored to obtain information regarding the disruption of the membrane by several conjugated bile salts. The results of their experiments demonstrated that hydrophilic bile salts protect membranes from damage by bile salts which are more hydrophobic (ie., taurodeoxycholate, taurochenodeoxycholate and taurocholate).[41] The results of their studies also indicated that the anion form of the bile salt species (rather than the protonated form) provided the protection.[41] This result implies that the pH of the hydrating solution may affect the interactions which bile salts have with membrane systems.

Guldutuna et al. also investigated the effects of UDC on hepatocyte membranes using electron paramagnetic resonance spectroscopy to monitor membrane structural changes.[44] The polarities of both the bile salt molecules and the various regions of the membrane were used to determine the location of the disturbance in the membrane caused by the interaction with the bile salt. CDC was found to alter the apolar core of the membrane and allow water to enter the membrane while UDC, when introduced into the lipid bilayer, was able to prevent any further disruption of the lipid bilayer from additional aliquots of CDC.[44]

Miyazaki et al. examined the effects of UDC and CDC on human hepatocytes, since these two bile salts have different side effects when administered to humans for the treatment of gallstones.[76] In these studies, the CDC epimer decreased the 'life span' of the hepatocyte to a greater extent, and at lower bile salt concentrations than did UDC.[76] The conjugated bile salts, while also decreasing the 'life span' of the hepatocytes, exhibited less of an effect than the unconjugated forms of the bile salts, with conjugated CDC

decreasing the 'life span' of the hepatocytes more than the UDC conjugated species.[76] Thus, conjugation appears to decrease the damaging properties that the unconjugated bile salts induce in hepatocytes.

Velardi et al. investigated the effects of phosphatidylcholine and cholesterol on bile salt damage in erythrocytes.[87] In these studies damage was measured by monitoring the release of hemoglobin from the erythrocytes. Results showed that the cells were damaged significantly when incubated with a solution containing only taurocholate. When the erythrocytes were incubated with a solution containing both taurocholate and phosphatidylcholine, the amount of damage done to the erythrocytes was reduced. Erythrocytes incubated with solutions of bile salt, phosphatidylcholine and cholesterol were found to have less damage than the cells incubated with the solutions containing either bile salt alone or binary mixtures containing bile salt and phosphatidylcholine.[87]

The effect which the pH of the hydrating solutions has on the interactions between bile salts and lipids must also be determined, since bile salts behave differently in solutions having different pH's.[28] At lower pH's, the unconjugated bile salt exists as the protonated form, while at higher pH's the conjugate base will predominate. Igimi and Carey have shown that protonated bile salts are more lipophilic.[28] Thus, the interaction of the bile salts with the membrane is likely to be pH dependent. The use of the unconjugated forms of the bile salts therapeutically also leads to questions concerning the effect which pH has on the interactions which these bile salts have with cellular membranes, since the pH of the gastrointestinal tract varies from 1.5 to 3.5 in the stomach and from 6.5 to 7.8 in the intestines.[88]

The effects which the unconjugated forms of two bile salt epimers have on model membranes composed of multilamellar vesicles of DPPC were determined. The effect which the hydrogen ion concentration of the hydrating solution has on the interactions of the bile salts with the DPPC bilayer was also investigated. Experiments were also performed to evaluate the spectral contribution of the bile salts to the methylene symmetric stretching band frequency, since the frequency of the methylene symmetric stretching mode is utilized to determine both conformational disorder and the perturbational effects which the addition of bile salts have on the bilayer.

2.2 Materials and Methods

2.2.1 Materials

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC, (manufacturer stated purity, > 99%) was purchased, in powder form, from Avanti Polar Lipids, Inc. (Alabaster, AL). DPPC from lot numbers 160PC-184, 160PC-187 and 160PC-188 was used for these studies. Sodium ursodeoxycholate (manufacturer stated purity, 99%, by TLC) and sodium chenodeoxycholate (manufacturer stated purity, > 97%, by TLC) were obtained from Calbiochem (La Jolla, CA). The bile salts were used as received. Potassium phosphate monobasic (KH_2PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Potassium hydroxide (KOH), in pellet form, was obtained from J.T. Baker Chemical Company (Pittsburgh, PA). Laboratory distilled, deionized water was used for the preparation of all solutions.

2.2.2 Sample Preparation

Stock solutions of 50 mM KH_2PO_4 were prepared by dissolving the appropriate mass of solid KH_2PO_4 in distilled, deionized water. The solutions were adjusted to a pH of 5, 7, or 8 using a concentrated potassium hydroxide solution. The stated solution pH is the initial pH of the hydrating solution, although the actual pH of the samples containing the bile salt is expected to be similar to or somewhat higher than the initial pH of the hydrating solution due to the complex acid-base and distribution equilibria which are established. Samples were prepared for analysis by weighing 0.1 grams of solid DPPC into a glass vial. The lipid was hydrated with 500 μl of the 50 mM KH_2PO_4 solution, previously adjusted to a pH of 5, 7, or 8, which corresponded to a 20 percent (w/v) concentration of DPPC. DPPC samples containing bile salts were prepared by adding the appropriate mass of solid DPPC and bile salt to the glass vial. The two solids were vortexed, before hydration with the appropriate KH_2PO_4 solution to ensure a homogeneous mixture of the solids. The bile salt containing samples were prepared to contain approximately 15 mole percent bile salt and 85 mole percent DPPC. Hydrated samples, in sealed vials, were heated above the DPPC gel to liquid crystalline phase transition temperature with the use of a water bath set at a temperature of approximately 59 °C. The samples were allowed to cool to room temperature while vortexing. This heating/cooling/vortexing process was repeated five times for each sample.

The solutions used in the experiment to determine the spectral contribution of the bile salt to the frequency of the lipid acyl chains' methylene symmetric stretching band were prepared as follows. The appropriate mass of solid CDC was dissolved in the 50

mM KH_2PO_4 stock solution, which had been previously adjusted to a pH of 7. The samples were vortexed to ensure complete mixing. The final concentrations of the sodium chenodeoxycholate solutions were: 0.0, 2.5, 5.0, 10.0, 20.0, 50.0, and 100.0 mM.

2.2.3 Data Acquisition

FTIR spectra were acquired using a Nicolet G-Series 520 bench (Nicolet Analytical Instruments, Inc., Madison, WI) equipped with a MCT-B detector and, OMNIC, a windows-based FTIR software package. All spectra were acquired at a nominal resolution of 2 cm^{-1} by coadding 100 scans. A 5 μl aliquot of the hydrated lipid was placed between two barium fluoride, BaF_2, (Spectra-Tech, Stamford, CT) or calcium fluoride, CaF_2, (Spectral Systems, Hopewell Junction, NY) windows using a 5 μm PTFE spacer (Goodfellow Corporation, Berwyn, PA). The use of a thin spacer was necessary due to the high concentration of water in the samples. The BaF_2 or CaF_2 windows were then placed into a thermoelectrically heated stage (BFS-30TC/ER, Physitemp Instruments, Inc., Clifton, NJ) which maintained the temperature of the sample to within ± 0.1 °C of the set temperature. Temperatures were monitored independently using an external thermocouple (BAT-12, Physitemp Instruments, Inc., Clifton, NJ) attached to the face plate of the thermoelectrically heated stage. Spectra were acquired at 1 °C intervals over a temperature range of 32 to 44 °C. The temperature was incremented using a program written in Visual Basic (Microsoft Corporation, Redmond, WA), which was interfaced to the FTIR data acquisition routines. The samples were allowed to equilibrate at each temperature for five minutes prior to acquiring spectral data. Single beam (ie., energy)

spectra were processed using MATLAB, a graphical analysis package (The Mathworks, Natick, MA). The spectra acquired for the lipid samples were corrected to account for the strong spectral contribution due to the water in the hydrating solution, prior to determining the frequency of the lipid's methylene symmetric stretching band. The correction procedure consisted of fitting the broad water absorption, in the 3050- 2650 cm^{-1} region, to a quartic function and then subtracting this feature from the C-H stretching mode region determined for the hydrated lipid. The frequency of the methylene symmetric stretching band was then determined by performing a center of gravity fit.[89] Thermotropic phase transition profiles for the bilayer assembly were obtained by plotting the frequency (cm^{-1}) of the lipid's methylene symmetric stretching band versus temperature (°C). The phase transition data were fit to a two-state model to obtain the phase transition temperatures and other parameters.[90]

A concern with these experiments involves the experimental reproducibility for determining the frequency of the methylene symmetric stretching band for independent samples. Based on data acquired in our laboratory and published levels of reproducibility for similar experiments, the frequency of the methylene symmetric stretching band for independent lipid samples can be determined within ± 0.1 cm^{-1}. This level of reproducibility is consistent with the levels established by Weers and Scheuing in their experiments on micellar sodium dodecylsulfate solutions.[91] The precision of the frequency of the methylene symmetric stretching mode in their experiments was determined to be within ± 0.04 cm^{-1}. Thus, for the data presented here, changes in the frequency of the methylene symmetric stretching band that are greater than ± 0.1 cm^{-1} are

considered significant.

The data for the experiments performed to determine the spectral contribution of the bile salt to the frequency of the methylene symmetric stretching bands were obtained in a similar manner as used for the DPPC samples containing bile salt. Briefly, data were first obtained for a DPPC sample hydrated with 50 mM KH_2PO_4 solution at an initial pH of 7 at two temperatures, 32 and 44 °C. These temperatures were chosen since they provide information on the lipid in the gel and the liquid crystalline phases, respectively. Once the spectra for DPPC were acquired, a sodium chenodeoxycholate solution was placed in a standard liquid cell consisting of two CaF_2 windows separated by a 25 μm PTFE spacer. The cell was placed on the source side, directly behind the thermoelectrically heated stage, which contained the DPPC sample, in the sample compartment. Spectra were then simultaneously acquired for the bile salt solution, which was at room temperature, and the DPPC sample at temperatures of 32 and 44 °C. Once the spectra for the DPPC sample with the CDC solutions were acquired, spectra for each of the bile salt solutions were obtained. That is, the DPPC sample was removed from the instrument and a spectrum was acquired for the bile salt solution alone. This procedure was repeated for each of the concentrations of CDC studied. The DPPC sample was never removed from the stage, but, the position of the stage was adjusted to allow the light reaching the detector to be maximized for each CDC solution studied. The spectral data were processed in the same manner used for all lipid samples. Absorption data were obtained by ratioing the spectra for the bile salt solutions to the spectrum of the 50 mM KH_2PO_4 hydrating solution.

2.3 Results and Discussion

The methylene stretching modes are generally the strongest bands in the infrared spectrum of hydrated lipid samples.[13, 14] The frequencies of these modes have been shown to be sensitive to the number of gauche conformers in the hydrocarbon chains of the lipids.[13, 14] As the number of gauche conformers in the hydrocarbon chains of the lipid increases, the frequency of the symmetric and asymmetric methylene stretching bands located at approximately 2850 cm^{-1} and 2920 cm^{-1}, respectively, shift to higher wavenumbers. An increase in the number of gauche conformers results in a more fluid, "disordered" bilayer assembly, while a decrease in the number of gauche conformers results in a less fluid, more "ordered" bilayer.[1, 4, 13] The main gel to liquid crystalline phase transition for the DPPC bilayer occurs at approximately 41 °C for the pure, hydrated lipid. This phase transition involves intrachain trans/gauche isomerization and expansion of the hydrocarbon lattice.[4] Intramolecular order/disorder chain packing characteristics are indicated by changes in the intensity and frequency of the carbon hydrogen stretching modes.[1] It has been estimated that approximately five gauche conformers are introduced into each of the acyl chains upon going through the gel to liquid crystalline phase transition.[14] The methylene symmetric stretching mode was monitored in these experiments due to its sensitivity to conformational changes, high intensity, and the minimal spectral overlap with the broad water absorption.

2.3.1 Experiment to Determine the Magnitude of the Spectral Contribution Due to the Presence of Bile Salt

The goal of this work was to utilize the shifts in the absorption frequencies of the lipid's acyl chain methylene stretching modes to indicate the extent of both conformational disorder and environmental perturbations occurring within the membrane bilayer due to the addition of bile salts. It is, therefore, important to establish the spectral effects in this region arising from the presence of the added bile salts on the frequency determined for the methylene symmetric stretching band of the lipid assembly. Basically, if the effect which a molecule has on the lipid assembly is to be determined and the molecule contains functional groups which absorb in the region being monitored for the lipid, the level of interference (ie., absorbance) from this molecule needs to be established. Experiments were performed to determine if the added bile salt affects the frequency of the lipid's methylene symmetric stretching band. In order to evaluate the magnitude of the effect, spectra were acquired for solutions of bile salt, at various concentrations. The spectral data were examined to determine the minimum concentration of bile salt, which due to spectral overlap results in a shift in the frequency of the lipid's methylene symmetric stretching band.

Several CDC containing solutions were used to evaluate the spectral contribution of the bile salts to the lipid modes. A plot of the absorbance of the methylene symmetric stretching band of the bile salt versus concentration of CDC in a 25 μm pathlength cell is given in Figure 2.2. This plot indicates the absorbance due to the bile salt in the 2850 cm^{-1} region is less than 0.05 for bile salt concentrations below 56 mM. As expected the

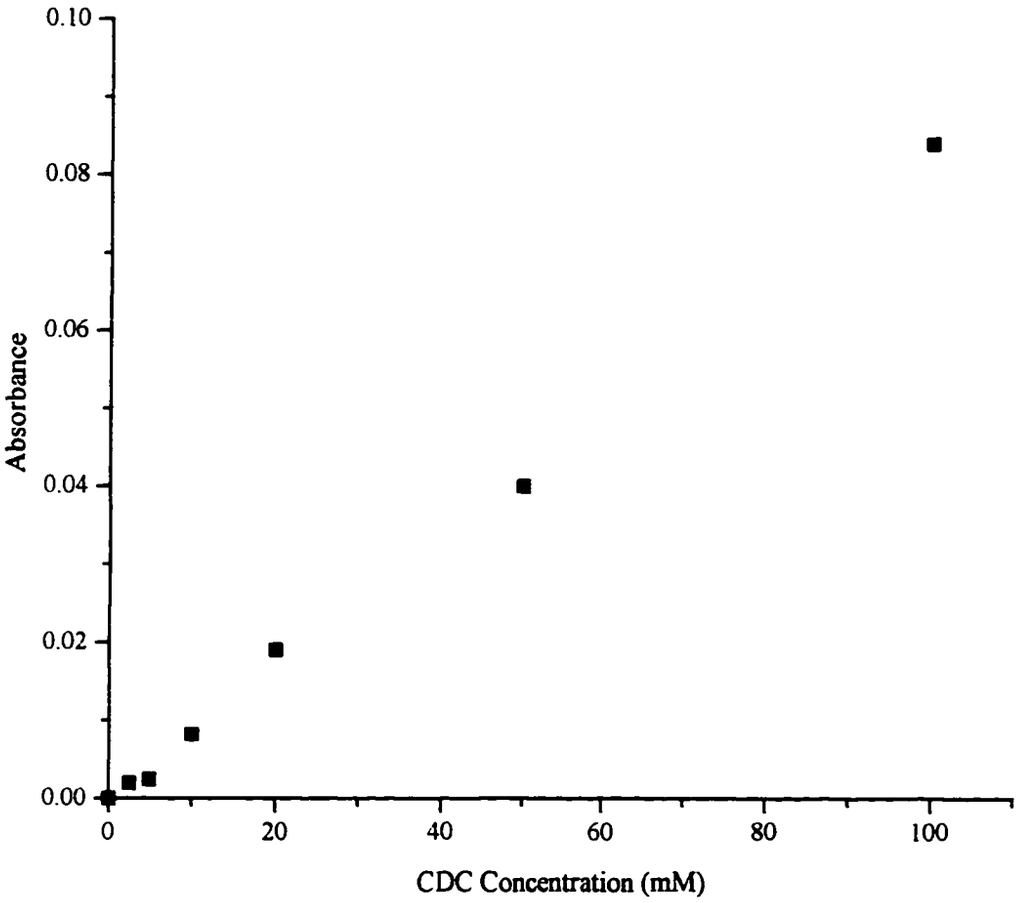


Figure 2.2: Absorbance of the Symmetric Methylene Stretching Band ($2850\ cm^{-1}$) for Several CDC Solutions versus Concentration of CDC

relationship between absorbance and concentration is linear. In order to compare the data obtained for studies which use bile salts at a concentration of 15 mole percent (85 mole % lipid) to the data obtained here, the solution concentrations must be converted to a concentration which is equivalent to the mole percent bile salt in the lipid sample. This calculation is performed by taking into consideration the pathlength, the concentration of the bile salt solution and the normal concentration of lipid in the lipid sample. The following equation was used for the conversion:

$$\text{mole\%} = (b_{BS})(\text{moles}_{BS})/[(b_{BS})(\text{moles}_{BS}) + (b_{DPPC})(\text{moles}_{DPPC})] * 100 \qquad (1),$$

where b_{BS} and b_{DPPC} are the pathlengths (ie., thicknesses of the spacers) of the cell containing the bile salt samples and the DPPC sample, respectively. The number of moles of DPPC and bile salt is designated in the equation as moles_{DPPC} and moles_{BS}, respectively. The results of these experiments show that for lipid samples containing the equivalent of fifteen mole percent bile salt, the absorbance, at 2850 cm^{-1}, due to sodium chenodeoxycholate is less than 0.005.

The effect which the spectral contribution of the bile salt, at various concentrations, has on the frequency of the lipid's methylene symmetric stretching band was determined. A plot of the methylene symmetric stretching band frequency for the DPPC bilayer versus the equivalent mole percent CDC is given in Figure 2.3. The data indicate that the frequency of the lipid's methylene symmetric stretching band for the CDC containing solutions having concentrations below 87 mole percent are within ± 0.1 cm^{-1} of

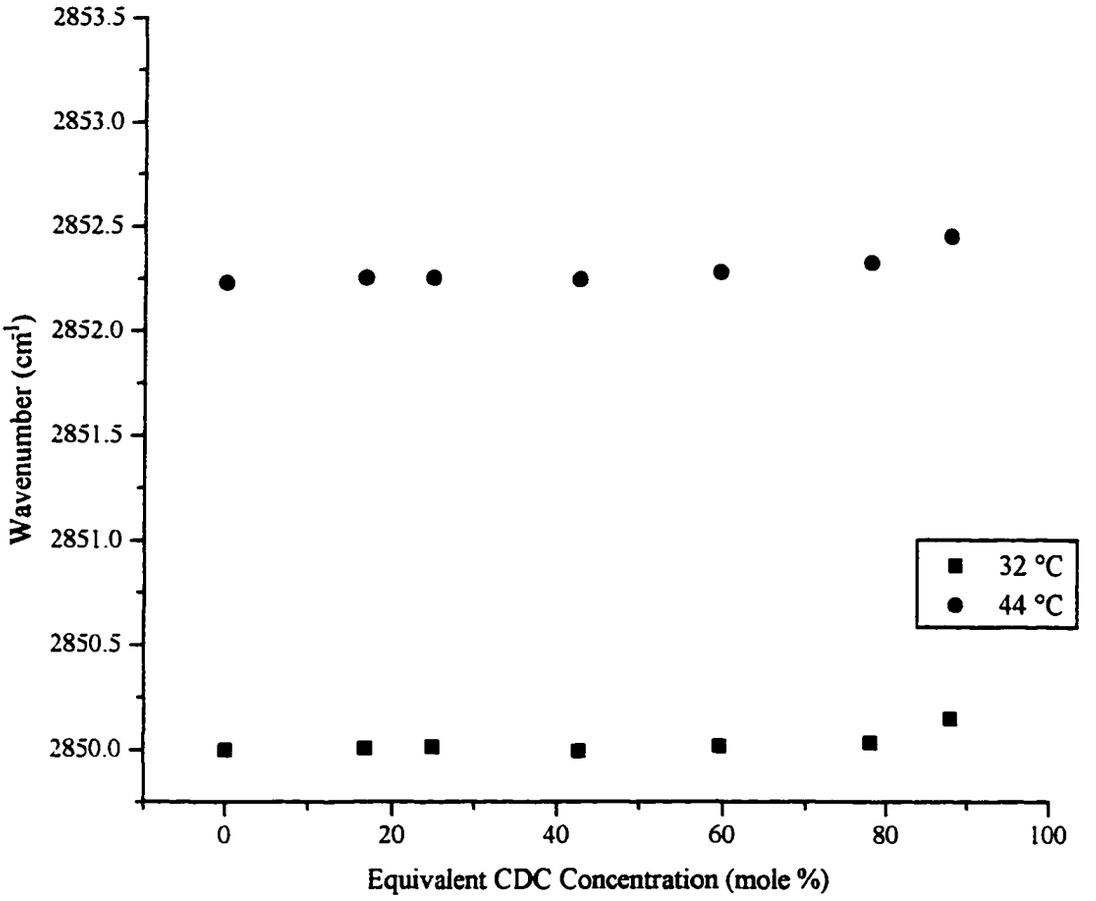


Figure 2.3: Symmetric Methylene Stretching Band Position for DPPC with CDC Solutions versus Mole Percent CDC

each other for both the gel and liquid crystalline phases. Thus, there is no significant change observed in the frequency of this band in either phase due to the presence of these solutions. The frequency of the lipid's methylene symmetric stretching band in the liquid crystalline phase is shifted, by 0.2 cm^{-1}, to higher wavenumbers at bile salt concentrations which are greater than approximately 87 mole percent bile salt. A shift in the frequency of the lipid's methylene symmetric stretching band of this magnitude is considered to be significant. There is, however, no significant change in the frequency of this band for the gel phase. Thus, the data indicate that at a bile salt concentration which is equivalent to 15 mole percent bile salt in lipid, the level used for the studies presented here, the spectral contribution of the bile salt does not affect significantly the frequency determined for the lipid's methylene symmetric stretching band.

2.3.2 Effect of Time

Studies were performed to determine the effects which UDC and CDC have on the DPPC bilayer over a period of time. Data were acquired for samples immediately after preparation ("fresh"), again after 24 hours, and 5 days after preparation. The samples were held at room temperature during the five days required for the experiments. Previous experiments had indicated that changes in the effects which these bile salts have on the bilayer may occur over time. No significant differences in the phase transition profiles were observed for the experiments presented here for samples after either 24 hours or 5 days. For this reason, data are presented for "fresh" samples only, (ie., data acquired within one hour after preparation).

2.3.3 Effect of Bile Salt

Studies were performed to determine the effects which the bile salts, UDC and CDC, at the fifteen mole percent level, have on the DPPC bilayer under various hydrating conditions. Phase transition profiles for samples of DPPC with and without the addition of approximately 15 mole percent of either bile salt, hydrated using a 50 mM KH_2PO_4 solution at an initial pH of 7 are given in Figure 2.4. The addition of bile salt does not significantly affect the frequency of the symmetric methylene stretching band for the lipid assembly in the gel state; that is, the change in the frequency of the band is less than ± 0.1 cm^{-1}. These data are interpreted to indicate that the addition of bile salt does not affect significantly the number of gauche conformers in the hydrocarbon chains of the lipid in the gel phase. The addition of UDC or CDC does, however, result in an increase in the frequency of the methylene symmetric stretching band for the lipid in the liquid crystalline phase under these hydrating conditions. The frequency of the methylene symmetric stretching band increases by approximately 0.5 cm^{-1} for the UDC containing sample and 0.6 cm^{-1} for the sample that contains CDC in comparison to pure DPPC. These results suggest that there is an increase in the number of gauche conformers in the acyl chains of the lipid in the liquid crystalline phase. Thus, these data indicate that the addition of the bile salt does not significantly affect the fluidity of the gel phase, but does significantly increase the fluidity of the bilayer in the liquid crystalline phase. The phase transition temperature decreases with the addition of either bile salt, at this concentration, relative to that of pure DPPC. The phase transition temperature decreases by 3.0 °C for the sample

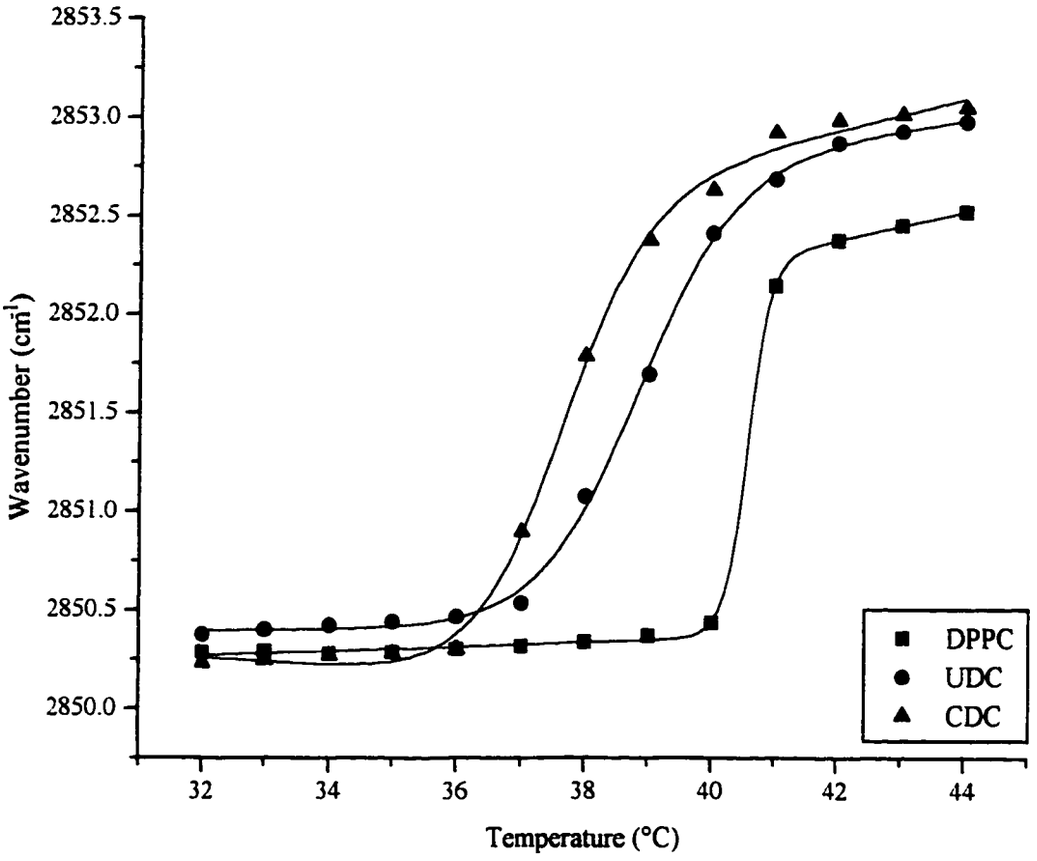


Figure 2.4: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 50 mM KH_2PO_4 Solution (Initial pH = 7)

which contains CDC, but only decreases by 1.8 °C for the sample which contains UDC. A decrease in phase transition temperature indicates that the presence of either bile salt destabilizes the bilayer structure. The profiles clearly show that the breadth of the phase transition of the bilayer assembly increases with the addition of either bile salt at the 15 mole percent level. The breadth increases to 3.1 °C for the DPPC sample that contains UDC, in comparison to the pure DPPC sample whose phase transition breadth is equal to 0.6 °C, while the increase in breadth for the CDC containing sample is smaller, than that obtained for the UDC containing sample, with a value of 2.8 °C. An increased phase transition breadth indicates a decrease in the cooperativity between the DPPC molecules in the bilayer during the phase transition. The breadths of the phase transitions and the phase transition temperatures for these samples are summarized in Table 2.1.

Experiments were performed to determine if the initial pH of the hydrating solution affects the interaction which the bile salts have with the DPPC bilayer. The phase transition profiles for samples of DPPC, DPPC with 15 mole percent UDC, and DPPC with 15 mole percent CDC, hydrated using a 50 mM KH_2PO_4 solution at an initial pH of 5, are given in Figure 2.5. The level of acyl chain disorder in the liquid crystalline phase increases for the bile salt containing samples, as can be observed by the increase in the frequency of the methylene symmetric stretching band. The methylene symmetric stretching band frequency increases by approximately 0.5 cm^{-1} for both bile salt containing samples. There does not appear, however, to be a significant change in the number of gauche conformers in the lipid's acyl chains in the gel phase for the CDC containing sample. The sample which contains UDC, however, does show an increase of 0.2 cm^{-1} in

Table 2.1: Breadths of the Phase Transitions (ΔT) and Phase Transition Temperatures (ΔT_m)

	pH	5	7	8
UDC				
	ΔT_m (°C)*	2.3	1.8	5.6[a], 0.7[b]
	ΔT (°C)	1.0	3.1	0.4[a], 5.7[b]
CDC				
	ΔT_m (°C)*	3.5	3.0	5.9[a], 1.2[b]
	ΔT (°C)	0.6	2.8	0.8[a], 4.5[b]

* Values equal to (T_{DPPC} - $T_{Bile\ Salt}$)
[a] Data acquired for the first phase transition
[b] Data acquired for the second phase transition

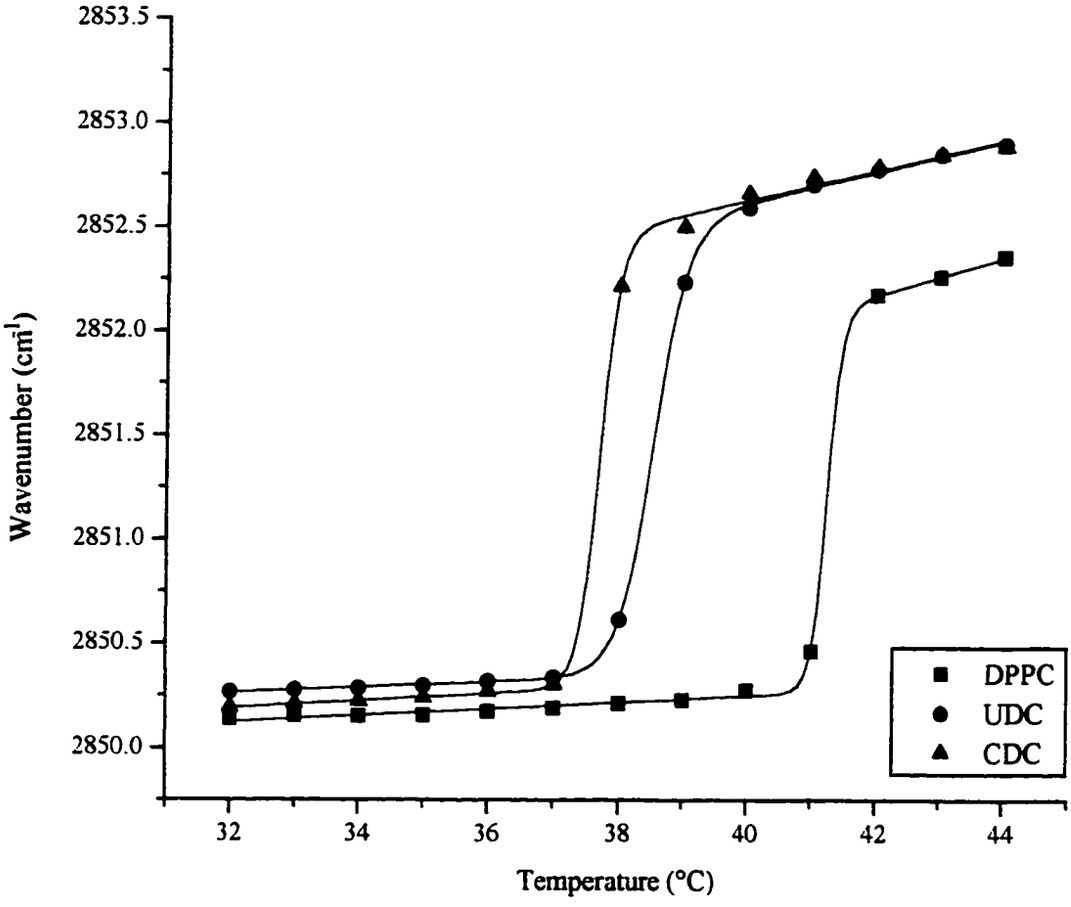


Figure 2.5: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 50 mM KH_2PO_4 Solution (Initial pH = 5)

the frequency of the methylene symmetric stretching band. The shift in the frequency, to higher wavenumbers, of the methylene symmetric stretching band indicates that the lipid's acyl chains for the sample containing CDC are more disordered in the gel phase. The presence of 15 mole percent of either UDC or CDC results in a decrease in the bilayer phase transition temperatures by up to 3.5 °C, which is similar to the phase transition temperatures obtained for samples containing bile salts but hydrated with a phosphate solution initially at a pH of 7. The breadths of the phase transition profiles are 0.5 °C for the pure DPPC sample, 1.0 °C for the UDC containing sample, and 0.6 °C for the CDC containing sample. Thus, the breadths of the phase transitions increase slightly with the addition of either bile salt which is quite different from the data acquired for the samples hydrated with a solution having an initial pH of 7. The breadths of the phase transitions and the phase transition temperatures are summarized in Table 2.1.

Studies were also carried out to determine the effects which a hydrating solution having a higher initial pH has on the bile salt/DPPC interactions. The phase transition profiles presented in Figure 2.6 show the effects that the addition of either bile salt has on the DPPC bilayer which was hydrated with a 50 mM KH_2PO_4 solution initially at a pH of 8. It is interesting to note that the phase transition profiles for the bile salt containing samples hydrated with a solution having an initial pH of 8 differ in their appearance from the samples hydrated with solutions having initial pH's of 5 or 7. There are two distinct regions in the phase transition profiles of the former samples. The profiles can be described as being biphasic. That is, there may be two types of phases present in the lipid assembly possibly corresponding to a region which is "bile salt-rich" and a second region

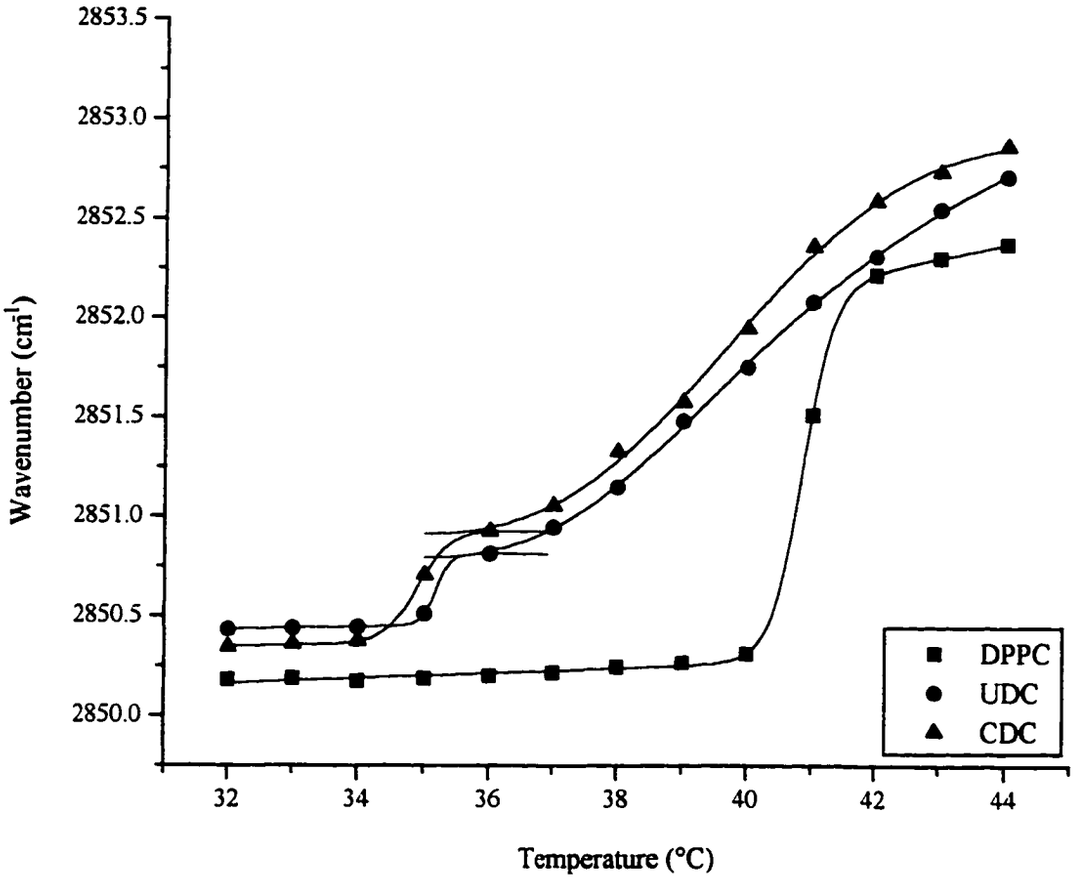


Figure 2.6: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 50 mM KH_2PO_4 Solution (Initial pH = 8)

that is "bile salt-poor." The frequency of the methylene symmetric stretching band indicates an increase in hydrocarbon chain disorder (ie., the frequency of the methylene symmetric stretching band has increased) in both the gel and liquid crystalline phases for the sample containing UDC. The frequency of the band increases by 0.2 cm^{-1} in the gel phase and by 0.3 cm^{-1} in the liquid crystalline phase. This change in the level of disorder in the lipid's hydrocarbon chains for the gel phase has not been observed for other bile salt containing samples. The frequency of the methylene symmetric stretching band for the liquid crystalline phase increases by 0.7 cm^{-1} for the CDC containing sample. The phase transition temperatures determined for the first phase transition for the UDC and CDC containing samples were 5.6 °C and 5.9 °C lower than for pure DPPC. The phase transition temperatures for the second phase transition for the UDC containing sample was 0.7 °C lower than pure DPPC while the phase transition temperature for the sample containing CDC decreased by 1.2 °C. The breadth of the first phase transition for the UDC and CDC containing samples are 0.4 °C and 0.8 °C, respectively. The breadths of the second phase transition are 5.7 °C and 4.5 °C for the UDC and CDC containing samples, respectively. The increase in the breadth of the second phase transition indicates that the cooperativity of the phase transition is much lower for the samples hydrated with the solution at an initial pH of 8 than those hydrated with solutions having initial pH's of 5 or 7. The breadths of the phase transitions and the phase transition temperatures are summarized in Table 2.1.

The data obtained also allow comparisons to be made for each type of sample (ie., pure DPPC, DPPC containing 15 mole percent UDC, and DPPC with 15 mole percent

CDC) and hydrating conditions studied. The phase transition profiles of the pure DPPC samples showed no significant differences when hydrated with 50 mM KH_2PO_4 solutions having initial pH's of 5, 7, or 8. Clearly, differences in the phase transition profiles were observed for the DPPC samples containing either bile salt when the samples were hydrated using solutions having initial pH's equal to 5, 7 or 8. The phase transition profiles for the samples containing either UDC or CDC hydrated with 50 mM KH_2PO_4 solutions having initial pH's of 5, 7, or 8 are given in Figures 2.7 and 2.8, respectively. The following trends were observed for the bile salt containing samples, the breadth of the phase transition increased as the pH of the hydrating solution increased and the bile salt containing samples hydrated with a KH_2PO_4 solution initially at a pH of 8 showed biphasic characteristics while no biphasic characteristics were apparent for samples hydrated with phosphate solutions initially at pH's of 5 or 7.

2.4 Conclusion

Shifts in the frequency of the methylene stretching bands of the lipid have been used widely to indicate changes in the ratio of gauche to trans conformers in the lipid's acyl chains. Recent studies by Kodati et al. suggest that caution should be used in interpreting such shifts as being due solely to changes in conformational order, suggesting other possible contributions to the band frequency including intermolecular coupling and librotorsional motion.[92] The results of their work also indicate that the effects of changes in the interchain vibrational coupling should be of less concern for the more fluid liquid crystalline phase than the less fluid gel phase of the lipid bilayer.[92] The results of

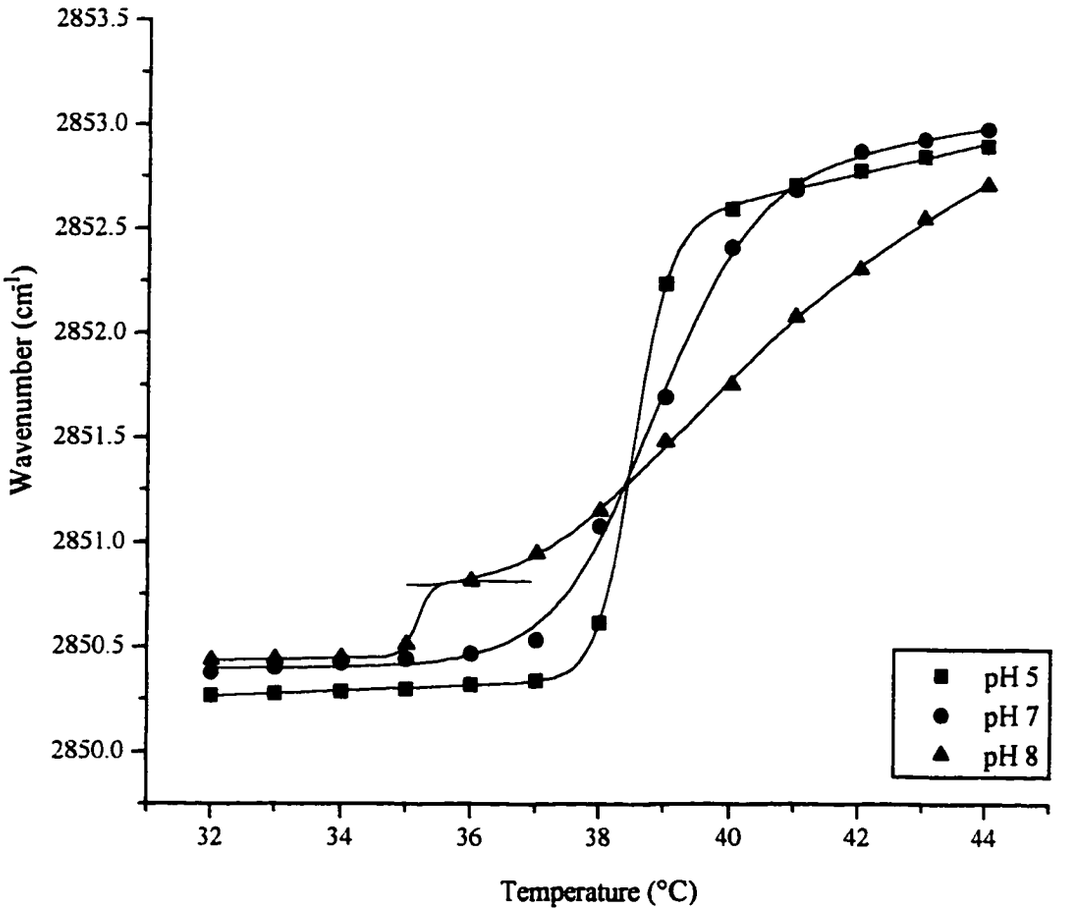


Figure 2.7: Phase Transition Profiles for DPPC/UDC Samples Hydrated with 50 mM KH_2PO_4 Solutions at Various pH's

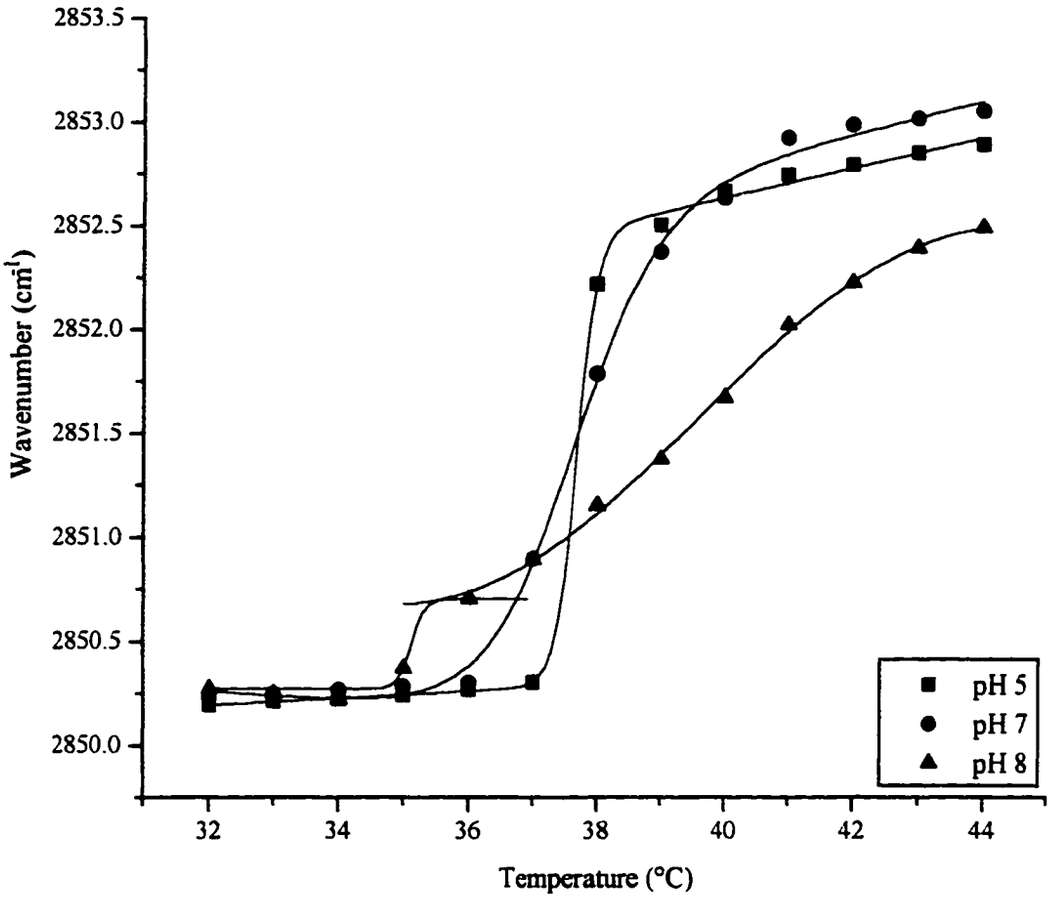


Figure 2.8: Phase Transition Profiles for DPPC/CDC Samples Hydrated with 50 mM KH_2PO_4 Solutions at Various pH's

our studies do not show appreciable effects of the bile salts on the methylene symmetric stretching band frequency in the gel phase. Thus, it is reasonable to expect that the perturbations which have been observed in the frequency of the methylene symmetric stretching band, are due primarily, though not exclusively, to conformational changes in the lipid's acyl chains.

The results of the experiments undertaken to determine the effect which the spectral contribution of the bile salt has on the frequency determined for the methylene symmetric stretching band show that the presence of the bile salts does not affect significantly the frequency determined for the methylene symmetric stretching band at concentrations below 87 mole percent bile salt in the lipid sample. At bile salt concentrations greater than 87 mole percent, the spectral contribution of the bile salt in the region of the methylene symmetric stretching band of the lipid does appear to interfere with the determination of the frequency of the band. This interference causes a shift in the frequency of the methylene symmetric stretching band to higher wavenumbers. The results of these experiments are reasonably consistent with the results reported by Kodati and Lafleur.[93] The results of their study indicate that the frequency of the methylene symmetric stretching band for the lipid's acyl chains is dependent on cholesterol concentration above cholesterol concentrations of 45 mole percent. The spectral features of cholesterol and the bile salts are similar in the methylene stretching region. Several possible explanations for the differences observed between the two sets of data can be postulated, including differences in the types of experiments performed, the data manipulation required for each type of experiment, and the lipid chosen for the two

studies. The studies presented here used a lipid assembly composed of DPPC whereas, Kodati et al. used 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) as the lipid system. Kodati and Lafleur performed a spectral subtraction to obtain their results rather than spectral addition which was used for this experiment.[93] The former method involves more spectral manipulation and computation than the latter technique. The increased spectral contribution due to the water in the aqueous bile salt solutions, which must be corrected for, can introduce errors in the spectral addition experiments presented here. The results from both sets of experiments, indicate that it is reasonable to believe the addition of the bile salts to the DPPC bilayer at concentrations which are less than approximately 40 mole percent do not significantly affect the frequency determined for the methylene symmetric stretching band of the lipid's acyl chains. Therefore, the results of these experiments provide confidence that shifts in the methylene symmetric stretching band frequency greater than 0.1 cm^{-1}, for samples containing fifteen mole percent of either bile salt, are in fact due to the interaction of the bile salts with the lipid assembly.

The addition of either unconjugated CDC or UDC, at the fifteen mole percent level, to DPPC multilamellar assemblies hydrated with a 50 mM KH_2PO_4 solution having initial pH's of 5, 7, or 8: 1.) destabilizes the bilayer which results in a lower phase transition temperature, 2.) increases the breadth of the phase transition, which indicates a reduction in the cooperativity between the lipid molecules during the phase transition and, 3.) reduces acyl chain disorder in the liquid crystalline phase suggesting an increase in the fluidity of this phase. Lesser effects are observed on the acyl chain order in the gel phase. The degree to which the bile salts affect the DPPC bilayer clearly is dependent on the

initial pH of the hydrating solution which supports theories suggesting the unprotonated and protonated forms of the bile acids have different effects on the lipid structure. That is, at higher pH's, where it is expected the unprotonated forms will predominate, there is greater effect on the breadth of the phase transition. At lower pH's where the protonated form of the bile salts will predominate, sharper phase transition profiles are observed for both the UDC and CDC containing samples.

Both UDC and CDC were found to destabilize the bilayer at the bile salt concentrations studied. The results show that the CDC epimer destabilizes the bilayer to a greater extent than does the UDC epimer based on the lower phase transition temperatures obtained for the CDC containing samples. A decrease in the stability of the bilayer with the addition of CDC is consistent with the results obtained by Scholmerich et al. [75] The authors performed experiments in which they monitored the release of tritiated inulin from large unilamellar liposomes prepared from egg lecithin. Other experiments were performed to monitor the release of an enzyme from rat hepatocytes. Both experiments indicate that exposure of the membranes to deoxycholate and chenodeoxycholate, in either the unconjugated, taurine conjugated or glycine conjugated form, resulted in an increase in the amount of tritiated inulin or enzyme released. The UDC epimer was found to be less effective at releasing the compounds than the CDC epimer. The authors also stated that cytotoxicity is inversely proportional to the degree of hydroxylation of the bile salt and that bile salts with a hydroxyl group in the 7 β position (UDC), destabilize the membrane to a lesser degree than observed for bile salts having a hydroxyl group in the 7 α position (CDC).

Many models have been proposed to explain how the bile salts interact with the lipid bilayer. Bayerl et al. studied the interactions of sodium deoxycholate with DPPC vesicles by FTIR spectroscopy.[42] They concluded that the FTIR data indicate that the bile salts interact at either of two sites, the aqueous interface region or the acyl chain region, depending on the conditions. They suggest, for samples containing a low concentration of bile salt, a shift in the phase transition temperature, without significant changes in the number of gauche conformers in either the gel or liquid crystalline phases, indicates an interaction at the aqueous interface region. While significant changes in the frequency of the methylene symmetric stretching band, indicating an ordering of lipid acyl chains in the liquid crystalline phase for samples containing high concentrations of bile salt, suggest the sodium deoxycholate molecules are interacting with the second site, the acyl chain region. The basis for this argument is a comparison of the data obtained for bile salt samples and others in which cholesterol is incorporated into the DPPC bilayer. Cholesterol is known to interact in the acyl chain region which has been reported to result in an ordering of the liquid crystalline phase, a disordering of the gel phase and an increase in the breadth of the phase transition.

Guldutuna et al. studied the interactions of CDC and UDC with red blood cells and hepatocyte membranes using EPR spectroscopy.[44] The results of their studies suggest that the unconjugated form of UDC interacts with the apolar core of the bilayer while the glycine and taurine conjugated forms of UDC interact with the polar interface region of the bilayer.

Heuman and Bajaj determined that the degree of protection provided by UDC

from membrane damage due to the more hydrophobic bile salts depends on the pH of the hydrating solution for the unconjugated form of the is bile salt.[41] They found that the protonated form of UDC, which predominates at lower pH's, provides less protection than the unprotonated form.

Clearly, care must be taken in comparing data between different studies since it is likely that even small variations in experimental conditions can change the complex bile salt/lipid interactions. The effects which the bile salts have on the lipid assembly will depend on the molar ratio of the bile salt to lipid, the concentration of bile salt in the sample, the presence of other species in the bilayer (ie., cholesterol, etc.) and the particular hydrating conditions employed (salt content, particular buffers, solution pH, etc.).

In our studies, the effects which the bile salts have on the bilayer are clearly different from that which has been observed with the incorporation of cholesterol into the DPPC bilayer. Cholesterol is known to interact with the apolar region of the bilayer corresponding with the lipid's acyl chains. Further, incorporation of cholesterol, broadens the gel to liquid crystalline phase transition profile. Though the breadth of the phase transition for bile salt samples hydrated at higher pH's is consistent with the bile salt inserted into the apolar region of the bilayer, as in the case of cholesterol, the levels of acyl chain order observed are not consistent with the data available for the models based on the incorporation of cholesterol. Further, the breadth of the phase transition profile decreases with decreasing pH and it is expected that the anion form of the bile salt, which should predominate at higher pH's, is less hydrophobic than the protonated form and, therefore, less likely to interact with the hydrophobic acyl chain region of the bilayer. Thus, under

the conditions studied, the data presented here are consistent with an interaction of UDC and CDC with the aqueous interface region of the lipid bilayer.

CHAPTER 3

INTERACTIONS OF BILE SALTS AND ETHANOL WITH MULTILAMELLAR VESICLES COMPOSED OF DPPC UNDER VARIOUS HYDRATING CONDITIONS

3.1 Introduction

Studies were conducted to determine the manner in which the two bile salt epimers, sodium ursodeoxycholate (UDC) and sodium chenodeoxycholate (CDC), affect the DPPC bilayer under specific hydrating conditions. Two concentrations of KH_2PO_4 in the hydrating solutions were utilized to determine if the concentration of the KH_2PO_4 affects the interactions of these bile salts with the DPPC bilayer. A higher KH_2PO_4 concentration provides more buffering capacity, at least at the lower pH's studied, than a lower concentration KH_2PO_4 solution. Thus, the pH of the hydrating solution may change, depending on the concentration of KH_2PO_4 in the hydrating solutions, when bile salt is present in the sample. The effect of the pH of the hydrating solution when bile salt is present in the sample is important to evaluate since it has been shown that bile salts behave differently in solutions of different pH.[5] At lower pH's, the unconjugated bile salts will be in their protonated form and at pH's higher than the pk_a, they will be in their ionic form.[28] Igimi and Carey have shown that the protonated bile salts are more lipophilic which suggests that they will be more likely to partition into the lipid bilayer.[28] Thus, it is not unreasonable to believe that the bile salts may interact with the lipid membrane

differently depending on the pH of the hydrating solution. Additionally, we were interested in determining if changes in the concentration of the counterion in the hydrating solution affects the interactions observed between the bile salt and the lipid bilayer. It has been shown that the concentration of the counterion (K^-) in the hydrating solution can affect the hydration of the head group which may lead to dehydration in this region of the lipid.[5, 94]

Experiments were also conducted using hydrating solutions containing various concentrations of ethanol to determine the effects which ethanol has on the bile salt/DPPC interactions. Ethanol is known to partition into the lipid bilayer which has been shown to cause changes in the physical properties of the lipid assemblies, thus altering membrane functions.[95] At low concentrations of ethanol in the hydrating solution, the gel to liquid crystalline phase transition temperature of the lipid bilayer decreases as the concentration of ethanol increases. The phase transition temperature eventually reaches a minimum at ethanol concentrations between 0.8 and 1.2 M (~41-62 mg/ml).[95] The acyl chains in the lipid bilayer are transformed from the "normal" noninterdigitated state of the gel phase to the interdigitated gel phase within this range of ethanol concentrations.[96, 97] The gel to liquid crystalline phase transition temperature of the lipid bilayer increases with additional increases in the ethanol concentration of the hydrating solution.[95, 96]

In the body, bile is secreted into the small intestines when the gall bladder contracts during digestion. The majority of ethanol that enters the body is absorbed in the small intestines.[98] Since bile salts and ethanol can both be present in the small intestines, studies which monitor the effect which bile salts have on the ethanol/model

membrane system may provide important clues to the understanding of the interactions which occur among the bile salts, ethanol, and cellular membranes in the body. By studying the interactions at ethanol concentrations which are both above and below the concentration known to induce interdigitation, the effects which bile salts have on a lipid assembly which is and is not interdigitated can be ascertained. The concentration of ethanol that is needed to induce interdigitation is high, but it has been determined that cells in the body may be exposed to these concentrations during periods of heavy drinking.[98] Therefore, studies performed on interdigitated bilayers are important in understanding the effects which ethanol can have on membranes in the human body.

Studies were performed to determine the effect which the addition of ethanol, at several concentrations, in the hydrating solution has on the interactions between the bile salts and the DPPC bilayer. Some ethanol experiments were performed using both infrared and Raman spectroscopies. This allowed for complementary data to be acquired. The data from the two experiments were compared and conclusions made regarding the intermolecular chain-chain disorder of the lipid's acyl chains and any conformational changes occurring within the lipid assembly. Experiments were also performed to evaluate the spectral contribution of the ethanol to the frequency of the methylene symmetric stretching band determined for the lipid assembly. Studies were conducted using two concentrations of KH_2PO_4 (50 mM and 200 mM) in the hydrating solutions to determine if the concentration of KH_2PO_4 affected the bile salt interactions with the bilayer. All studies were performed using hydrating solutions having initial pH's of 5, 7, and 8.

3.2 Materials and Methods

3.2.1 Materials

Synthetic 1,2 dipalmitoyl-sn-glycero-3-phosphocholine, DPPC, (manufacturer stated purity > 99%) was purchased, in powder form, from Avanti Polar Lipids, Inc. (Alabaster, AL). DPPC from lot numbers: 160PC-184, 160PC-187 and 160PC-188 was used for the experiments with 50 mM KH_2PO_4 hydrating solutions. DPPC from lot number 160PC-187 was used for the experiments with 200 mM KH_2PO_4 hydrating solutions. Sodium ursodeoxycholate, UDC, (manufacturer stated purity 99%, by TLC) and sodium chenodeoxycholate, CDC, (manufacturer stated purity > 97%, by TLC) were obtained from Calbiochem (La Jolla, CA). Bile salts were used as received without further purification. Fully deuterated ethanol (ethanol-d_6, manufacturer stated purity 99+ D %) was purchased from Sigma Chemical Company (St. Louis, MO). Fully deuterated ethanol was utilized to avoid spectral interference in the hydrocarbon region. Potassium phosphate monobasic (KH_2PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Potassium hydroxide (KOH), in pellet form, was obtained from J.T. Baker Chemical Company (Pittsburgh, PA). Laboratory distilled, deionized water was used for the preparation of all solutions.

3.2.2 Sample Preparation

The procedures used to prepare the lipid samples hydrated with KH_2PO_4 solutions having initial pH's of 5, 7, and 8 were similar to those described in Chapter 2. The aqueous stock solutions, however, were prepared to have KH_2PO_4 concentrations of 50 or

200 mM.

Hydrated lipid samples were also prepared, as described in Chapter 2, using stock solutions containing appropriate quantities of ethanol-d_6. The ethanol-d_6 containing solutions were prepared by pipetting appropriate masses of ethanol-d_6 into 5 ml volumetric flasks. The ethanol-d_6 was diluted to the mark using either 50 or 200 mM KH_2PO_4 stock solution with pH's previously adjusted to 5, 7, or 8. Solutions were prepared to contain 20, 55 or 120 mg of ethanol-d_6 per milliliter. It should be noted that the concentration of KH_2PO_4 in the hydrating solutions containing ethanol-d_6 is lower than the stated concentration. This is due to the solution being diluted with the addition of ethanol-d_6.

Solutions used in the experiment to determine the spectral contribution of the ethanol-d_6 on the frequency determined for the lipid's methylene symmetric stretching band were prepared in the following manner. An appropriate mass of deuterated ethanol was dissolved in 50 mM KH_2PO_4 stock solution, which had been previously adjusted to a pH of 7. The final concentrations of ethanol-d_6 in the solutions were: 4, 12, and 24 milligrams per milliliter.

3.2.3 Data Acquisition

The FTIR data were acquired and analyzed as described in Chapter 2.

Experiments were performed to determine if the spectral contribution of the deuterated ethanol in the hydrating solution affects the frequency determined for the lipid's symmetric methylene stretching band. Briefly, data were first obtained for a DPPC sample

hydrated using a 50 mM KH_2PO_4 stock solution at an initial pH of 7. Spectral data were acquired for the lipid sample in the gel and liquid crystalline states, at 32 and 44 °C, respectively. Once the spectra for the pure, hydrated DPPC sample were acquired at these temperatures, an aliquot of one of the ethanol-d_6 solutions was placed in a standard liquid cell consisting of two CaF_2 windows separated by a 25 μm PTFE spacer. The liquid cell was placed into the sample compartment between the source and the thermoelectrically heated stage which contained the DPPC sample. Thus, the infrared beam first passed through the ethanol-d_6 solution then through the lipid dispersion. Spectral data were acquired using this configuration for the lipid sample, at both 32 and 44 °C, and each of the ethanol-d_6 solutions (0, 4, 12, and 24 mg/ml, at room temperature). Once the spectra for the DPPC sample and the ethanol-d_6 solutions were acquired, spectra were acquired for each of the ethanol-d_6 solutions alone. This procedure was repeated for each of the ethanol-d_6 concentrations studied. The DPPC sample was not removed from the stage, though the thermoelectrically heated stage was removed from the instrument, to acquire the data for the ethanol-d_6 solutions. Each time the heated stage was placed into the instrument, it's position was adjusted to maximize the light reaching the detector. The spectral data were processed in the same manner used for all lipid samples.

Raman spectra were acquired, in the laboratory of Dr. Ira W. Levin at the National Institutes of Health in Bethesda, Maryland, using a Spex Ramalog 6 (Spex Industries Inc., Edison, NJ) dispersive spectrometer equipped with holographic gratings (188 grooves/mm). The laser excitation was provided by a Coherent Innova 100 argon ion laser (Coherent, Inc., Santa Clara, CA). The power at the sample ranged from 100 to 300

mW. The detector was a RCA C31034 photomultiplier tube. An aliquot of the multilamellar lipid assembly was placed into a glass capillary tube using a syringe. The sample in the capillary tube was centrifuged for 30 minutes to force the lipid to the end of the tube. The capillary was flame sealed after centrifugation. Spectra were acquired for the hydrocarbon stretching region at temperatures of 25, 32, 44 and 48 °C. The temperature was regulated by placing the sample in a thermoelectrically controlled sample holder. A personal computer was used to control the Raman spectrometer, control the sample temperature and record the Raman scattering. Spectra were acquired at a resolution of 5 cm^{-1}. Spectra were processed using LAP, a laboratory analysis package, to obtain peak intensities and peak intensity ratios (I_{2850}/I_{2880} and I_{2935}/I_{2880}).

3.3 Results and Discussion

The effects which the two, unconjugated bile salt epimers, UDC and CDC, have on the DPPC bilayer under various hydrating conditions were evaluated over time. Data were acquired for samples immediately after preparation ("fresh"), after 24 hours and again 5 days after preparation. The samples were held at room temperature during the five days required for the experiments. No significant differences were observed in the phase transition profiles obtained for these samples studied after either 24 hours or 5 days. For this reason, the data presented are limited to the "fresh" samples which were acquired within an hour after preparation.

3.3.1 Effect of Bile Salt on DPPC Multilamellar Assemblies Hydrated with 200 mM KH_2PO_4 Solutions Having Initial pH's of 5, 7, and 8

Experiments were performed to determine the effects which the bile salts, at the 15 mole percent level, have on the DPPC bilayer assemblies under several hydrating conditions. A summary of the phase transition temperatures relative to pure DPPC (ΔT_m), the methylene symmetric stretching frequencies and the breadths (ΔT) of the phase transitions for samples of DPPC with and without the addition of the bile salts using hydrating solutions having a KH_2PO_4 concentration of 200 mM and an initial pH of 7 is given in Table 3.1. The data show that the addition of either UDC or CDC, at a concentration of 15 mole percent, results in a shift in the frequency of the symmetric methylene stretching band of the lipid's acyl chains for the bilayer in both the gel and the liquid crystalline phases. The shift to higher wavenumbers indicates that addition of either bile salt increases the level of disorder of the lipid's hydrocarbon chains. The frequency of the methylene symmetric stretching band for the bile salt containing samples increases by approximately 0.2 cm^{-1} for the gel phase and by approximately 0.7 cm^{-1} for the liquid crystalline phase. This increase in lipid acyl chain disorder indicates that the addition of the bile salts causes the gel and the liquid crystalline phases to be more fluid than pure DPPC in the corresponding phase. The phase transition temperature for the lipid bilayer decreases when either bile salt is added, at the 15 mole percent level. The gel to liquid crystalline phase transition temperature of the DPPC bilayer decreases by 2.4 °C for the UDC containing sample and by 2.9 °C for the sample that contains CDC, compared to the phase transition temperature obtained for pure DPPC hydrated under similar conditions.

Table 3.1: Phase Transition Parameters for Samples Hydrated with 200 mM KH_2PO_4 Solutions (Initial pH = 7)

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2849.9	2849.9	2850.0
	44 °C*	2852.1	2852.3	2853.0
	ΔT (°C)	0.6	0.7	0.5
	ΔT_m (°C)**		0.9	0.1
UDC	32 °C*	2850.3	2850.2	2850.0
	44 °C*	2852.8	2853.0	2853.1
	ΔT (°C)	6.2	1.0	0.6
	ΔT_m (°C)**	2.4	2.6	1.0
CDC	32 °C*	2850.2	2850.2	2850.0
	44 °C*	2853.0	2852.9	2853.1
	ΔT (°C)	5.3	2.4	0.6
	ΔT_m (°C)**	2.9	3.1	1.1

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC\ (0\ mg/ml\ d6\text{-}ETOH)} - T_{Bile\ Salt}$)

The addition of either bile salt epimer, at this concentration, also results in an increase in the breadth of the phase transition compared to pure DPPC. Thus, the presence of the bile salt results in a decrease in the cooperativity between the lipid molecules of the bilayer during the gel to liquid crystalline phase transition. The breadth of the phase transition increases from 0.6 °C for pure DPPC to 5.3 °C for the CDC containing sample and 6.2 °C for the UDC containing sample.

These results are similar to the results obtained for the UDC and the CDC containing samples hydrated with a 50 mM KH_2PO_4 solution having an initial pH of 7. That is, the addition of either bile salt to the DPPC sample results in a decrease in the cooperativity between the lipid molecules during the phase transition, a destabilization of the lipid bilayer and an increase in the fluidity of the liquid crystalline phase. One difference was observed, however, between the data obtained using the 50 mM KH_2PO_4 hydrating solution and the 200 mM KH_2PO_4 hydrating solution. For the bile salt containing samples which were hydrated using a 50 mM KH_2PO_4 solution, initially at a pH of 7, the level of disorder of the lipid's acyl chains in the gel phase did not change from that observed for pure DPPC whereas the level of acyl chain disorder did change for the bile salt containing samples hydrated with the 200 mM KH_2PO_4 solution.

The effect which lowering the initial pH of the hydrating solution has on the interactions observed between these bile salts and the DPPC bilayer was also investigated. The frequency of the methylene symmetric stretching band for the lipid's acyl chains, ΔT_m's and ΔT's for DPPC, DPPC with 15 mole percent UDC and DPPC with 15 mole percent CDC, hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 5, are

given in Table 3.2. The effect which the bile salts, under these hydrating conditions, have on the level of lipid acyl chain disorder in the liquid crystalline phase is greater than in the gel phase. For example, the frequency of the methylene symmetric stretching mode for the UDC containing sample increases by 0.3 cm^{-1} in the gel phase and by 0.6 cm^{-1} in the liquid crystalline phase. The phase transition temperatures decrease by 3.5 °C and by 4.2 °C for the UDC and CDC containing samples, respectively, when compared to pure DPPC. The effect which the bile salts have on the breadth of the phase transition for samples hydrated with a solution having an initial pH of 5 is much less than was observed for the samples hydrated with a solution having an initial pH of 7. Spectra were acquired using both increasing and decreasing temperature ramps for the UDC containing sample hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 5. The phase transition profiles are presented in Figure 3.1. Only slight variations were observed in the frequency of the methylene symmetric stretching band for the gel and liquid crystalline phases. The profiles are similar but slight changes were observed in the cooperativity between the lipid molecules during the gel to liquid crystalline phase transition and in the phase transition temperature of the lipid bilayer.

Results obtained for samples hydrated with a 50 mM KH_2PO_4 solution having an initial pH of 5 are similar to those observed for the samples hydrated with a 200 mM KH_2PO_4 solution having a similar initial pH. One difference which does exist in the two sets of data is in the frequency of the methylene symmetric stretching band for the CDC containing sample. The frequency of the methylene symmetric stretching band for the lipid in the gel phase for the sample hydrated with a 50 mM KH_2PO_4 solution did not change

Table 3.2: Phase Transition Parameters for Samples Hydrated with 200 mM KH_2PO_4 Solutions (Initial pH = 5)

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2849.9	2849.9	2850.0
	44 °C*	2852.0	2852.3	2853.0
	ΔT (°C)	0.4	0.5	0.4
	ΔT_m (°C)**		0.9	-0.1
UDC	32 °C*	2850.2	2850.2	2850.2
	44 °C*	2852.6	2852.7	2853.2
	ΔT (°C)	1.0	1.3	0.2
	ΔT_m (°C) **	3.5	2.5	1.1
CDC	32 °C*	2850.1	2850.1	2850.2
	44 °C*	2852.8	2852.8	2853.2
	ΔT (°C)	1.8	0.6	0.6
	ΔT_m (°C)**	4.2	3.7	2.4

* Values in wavenumbers (cm^{-1})

**Values equal to ($T_{DPPC\ (0\ mg/ml\ d6\text{-}ETOH)} - T_{Bile\ Salt}$)

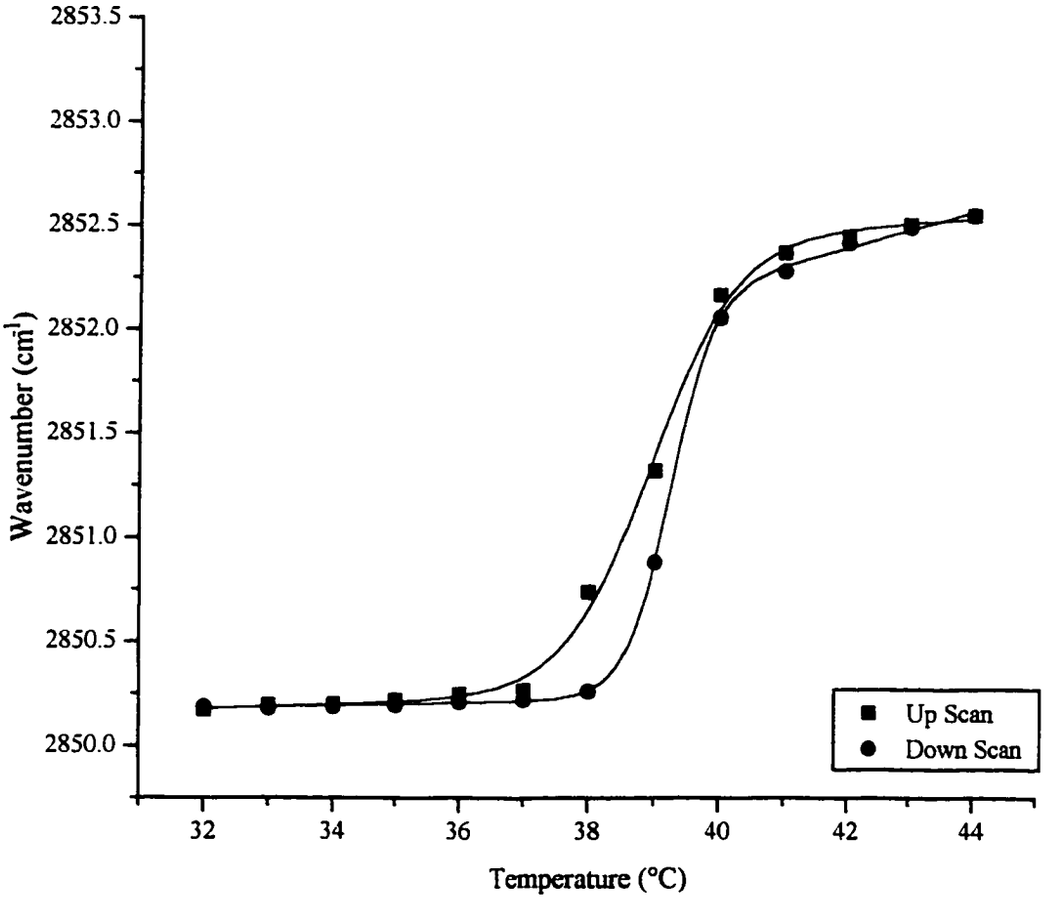


Figure 3.1: Phase Transition Profiles for DPPC/UDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 5)

compared to the frequency obtained for pure DPPC hydrated under similar conditions. The CDC containing sample hydrated with a 200 mM KH_2PO_4 solution did show an increase, 0.2 cm^{-1}, in the frequency of the methylene symmetric stretching mode, for the gel phase, compared to the pure DPPC hydrated with a similar solution.

To determine the effects of hydrating the bile salt/lipid samples with solutions having higher initial pH's, the experiments were repeated using 200 mM KH_2PO_4 hydrating solutions having an initial pH of 8. A summary of the methylene symmetric stretching band frequencies of the lipid's acyl chains, ΔT_m's and ΔT's for samples of DPPC with and without the addition of the bile salts hydrated using 200 mM KH_2PO_4 solutions with an initial pH of 8 is given in Table 3.3. Increases in the level of disorder of the hydrocarbon chains in the gel and the liquid crystalline phases were observed for the sample containing UDC. No change in the level of disorder of the acyl chains in the gel phase was observed for the sample containing CDC. The CDC containing sample does, however, show an increase in the level of lipid acyl chain disorder in the liquid crystalline phase. Thus, these data suggest the fluidity increases in the gel and the liquid crystalline phases for the UDC containing sample while the fluidity only increases in the liquid crystalline phase for the CDC containing sample. The phase transition profiles, given in Figure 3.2 for samples hydrated with this solution show an interesting phenomenon occurring at approximately 34 °C. The phase transition profiles obtained appear to be composed of two distinct phase transition regions. This may suggest that the bilayer consists of two domains possibly corresponding to a region which is "bile salt-poor" and a second region that is "bile salt-rich". Similar effects were observed in experiments

Table 3.3: Phase Transition Parameters for Samples Hydrated with 200 mM KH_2PO_4 Solutions (Initial pH = 8)

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2850.0	2849.8	2850.1
	44 °C*	2852.2	2852.1	2853.1
	ΔT (°C)	0.6	0.6	0.4
	T_m (°C)**		1.1	0.1
UDC	32 °C*	2850.4	2850.1	2850.0
	44 °C*	2852.5	2852.6	2853.0
	ΔT (°C)	0.4[a], 4.0[b]	1.9	0.2
	T_m (°C)**	6.9[a], 1.0[b]	2.6	0.4
CDC	32 °C*	2850.0	2850.0	2850.0
	44 °C*	2852.5	2852.7	2853.2
	ΔT (°C)	0.5[a], 5.9[b]	1.9	0.6
	T_m (°C)**	7.0[a], 1.9[b]	3.6	1.2

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC\ (0\ mg/ml\ d6\text{-}ETOH)} - T_{Bile\ Salt}$)

[a] Data acquired from the first phase transition

[b] Data acquired from the second phase transition

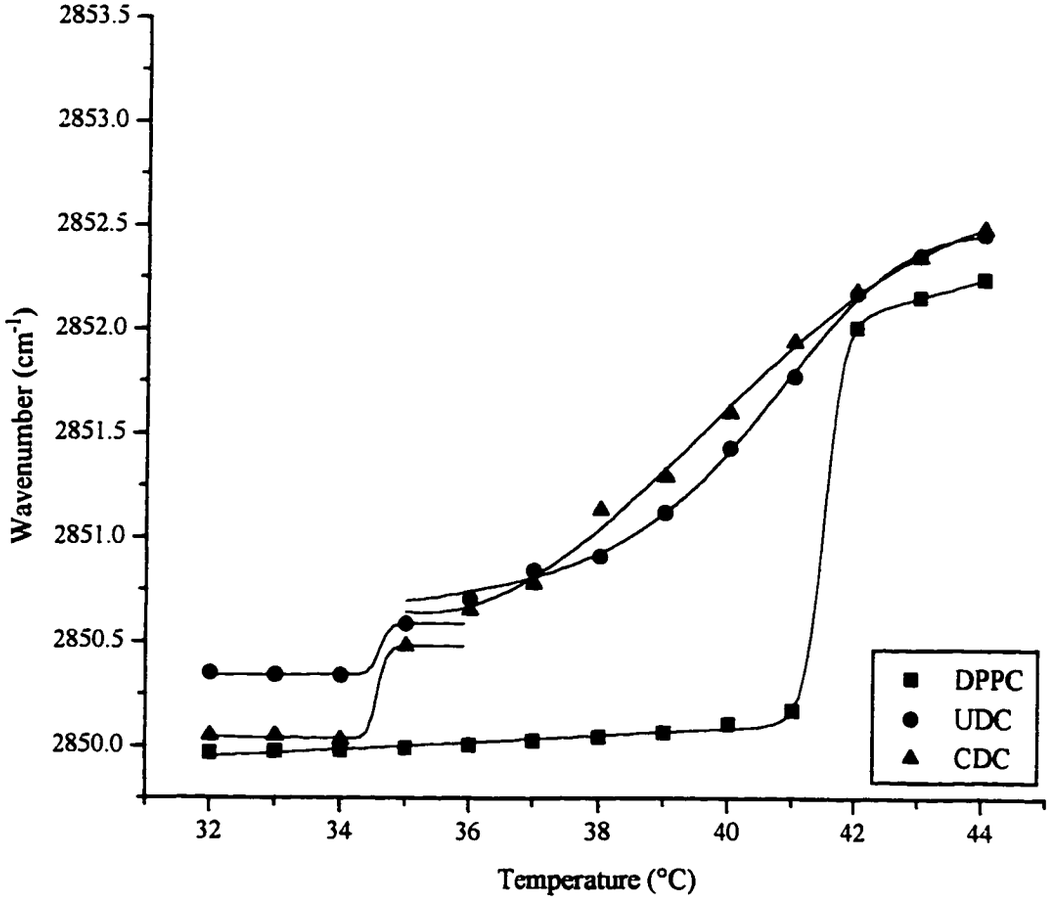


Figure 3.2: Phase Transition Profiles of DPPC/Bile Salt Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 8)

performed by McMullen et al. where cholesterol was incorporated into the lipid bilayer.[99] The phase transitions occur at 34.4 °C and 40.5 °C for the UDC containing sample and at 34.5 °C and 39.6 °C for the CDC containing sample. The breadths of the two phase transitions are very different. The lower temperature phase transition is sharp while the higher temperature phase transition is broad. The difference in the breadths of the two phase transitions indicates the cooperativity of the lipid molecules during the phase transition occurring at the higher temperature is lower than the phase transition at the lower temperatures.

It should be noted that the effects which the bile salts have on DPPC bilayer hydrated with a 50 mM KH_2PO_4 solution having an initial pH of 8 are comparable to those obtained for samples hydrated with a solution containing 200 mM KH_2PO_4 and having a similar pH.

3.3.2 Experiments to Determine the Effect which the Presence of Ethanol-d_6 in the Hydrating Solution has on the Frequency of the Lipid's Methylene Symmetric Stretching Band

Prior to interpreting the results which the presence of deuterated ethanol has on the lipid bilayer and on the bile salt interactions with the lipid bilayer, it is necessary to establish whether or not the presence of deuterated ethanol in the hydrating solution affects the frequency determined for the methylene symmetric stretching band of DPPC due to spectral interferences. These experiments must be performed due to the high concentration of ethanol-d_6 in some of the hydrating solutions. The presence of

deuterated ethanol may affect the underlying water absorption band or contribute spectrally to the frequency of the desired bands. A plot of the symmetric methylene stretching band peak frequency versus ethanol-d_6 concentration is given in Figure 3.3. It should be noted that this plot contains data for the deuterated ethanol solutions plus the DPPC samples. The concentrations of ethanol used for these experiments are five times lower than the concentrations on the x-axis of the plot due to the fact that the liquid cell used in these experiments had a pathlength of 25 μm while the DPPC sample cell's pathlength was 5 μm. Using this approach allowed for a comparison to be made between the data acquired here and the data acquired in later experiments using a bile salt concentration of 15 mole percent (85 mole % lipid). The data show that the change in the frequency determined for the methylene symmetric stretching band of the lipid for either the gel or the liquid crystalline phase is less than 0.1 cm^{-1} over the range of ethanol-d_6 concentrations studied. Thus, the results indicate that at the ethanol-d_6 concentrations used in these studies (0 to 120 mg/ml), changes observed in the frequency of the methylene symmetric stretching band, at levels greater than 0.1 cm^{-1} can be attributed confidently to effects which the ethanol and bile salts have on the DPPC bilayer.

3.3.3 Determination of the Effect of Adding Ethanol-d_6 to Hydrating Solutions Having a KH_2PO_4 Concentration of 50 mM on Bile Salt/Lipid Interactions

Experiments were performed to compare the effect which the presence of ethanol-d_6 in the hydrating solution has on the DPPC bilayer for samples with and without added bile salt when hydrated using 50 mM KH_2PO_4 solutions having an initial pH of 7. The

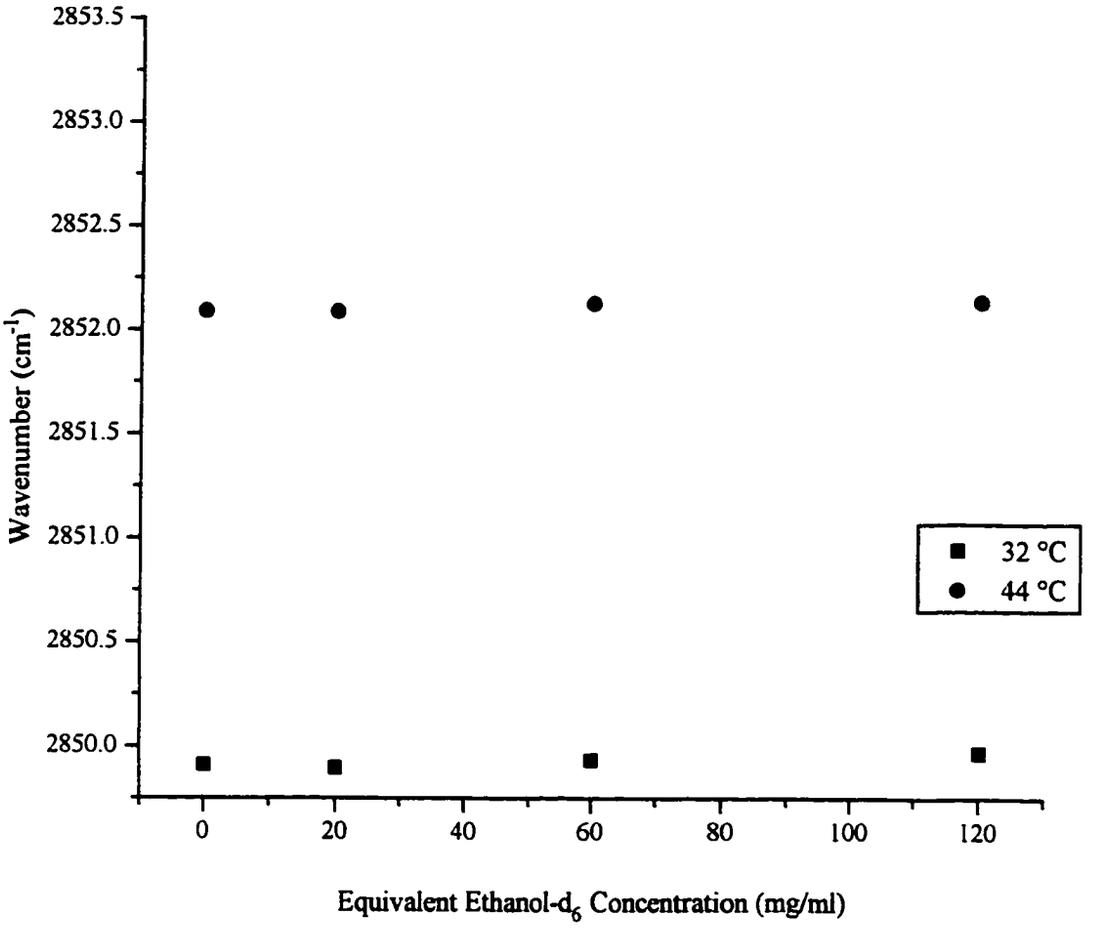


Figure 3.3: Symmetric Methylene Stretching Band Frequency for DPPC with Ethanol-d_6 Solutions Versus Equivalent Ethanol-d_6 Concentration

phase transition profiles for pure DPPC, DPPC containing 15 mole percent UDC and DPPC containing 15 mole percent CDC hydrated with a 50 mM KH_2PO_4 solution (initial pH = 7) containing 20 mg/ml ethanol-d_6 are given in Figure 3.4. The presence of 20 mg/ml ethanol-d_6 in the hydrating solution of samples of pure DPPC and DPPC with 15 mole percent UDC or CDC does not significantly affect the lipid acyl chain order in the gel phase or the liquid crystalline phase when compared to the data acquired for similar samples hydrated with a solution containing no ethanol. The phase transition temperatures for pure DPPC hydrated with a solution containing 20 mg/ml ethanol-d_6 decreases by 0.2 °C compared to pure DPPC hydrated with a solution that does not contain any ethanol-d_6. The phase transition temperature of the UDC containing sample hydrated with a 20 mg/ml ethanol-d_6 containing solution decreases by 0.7 °C compared to a similar UDC sample hydrated with a solution which does not contain ethanol-d_6. The phase transition temperature for the CDC containing sample hydrated with a solution having an ethanol-d_6 concentration of 20 mg/ml is different from that obtained for the CDC containing sample hydrated with a solution which does not contain ethanol-d_6. As can be seen by comparing the breadths of the phase transitions for samples hydrated with 50 mM KH_2PO_4 solutions containing 0 and 20 mg/ml ethanol-d_6, the breadths of the phase transitions for either of the bile salt containing samples decrease with the addition of even low concentrations of ethanol-d_6. The breadths for the DPPC samples containing the bile salts and hydrated with a solution which contains 20 mg/ml ethanol-d_6 decrease, from 3.1 °C to 2.8 °C for the UDC containing sample and, from 2.8 °C to 0.7 °C for the CDC containing sample. The phase transition parameters are given in Table 3.4.

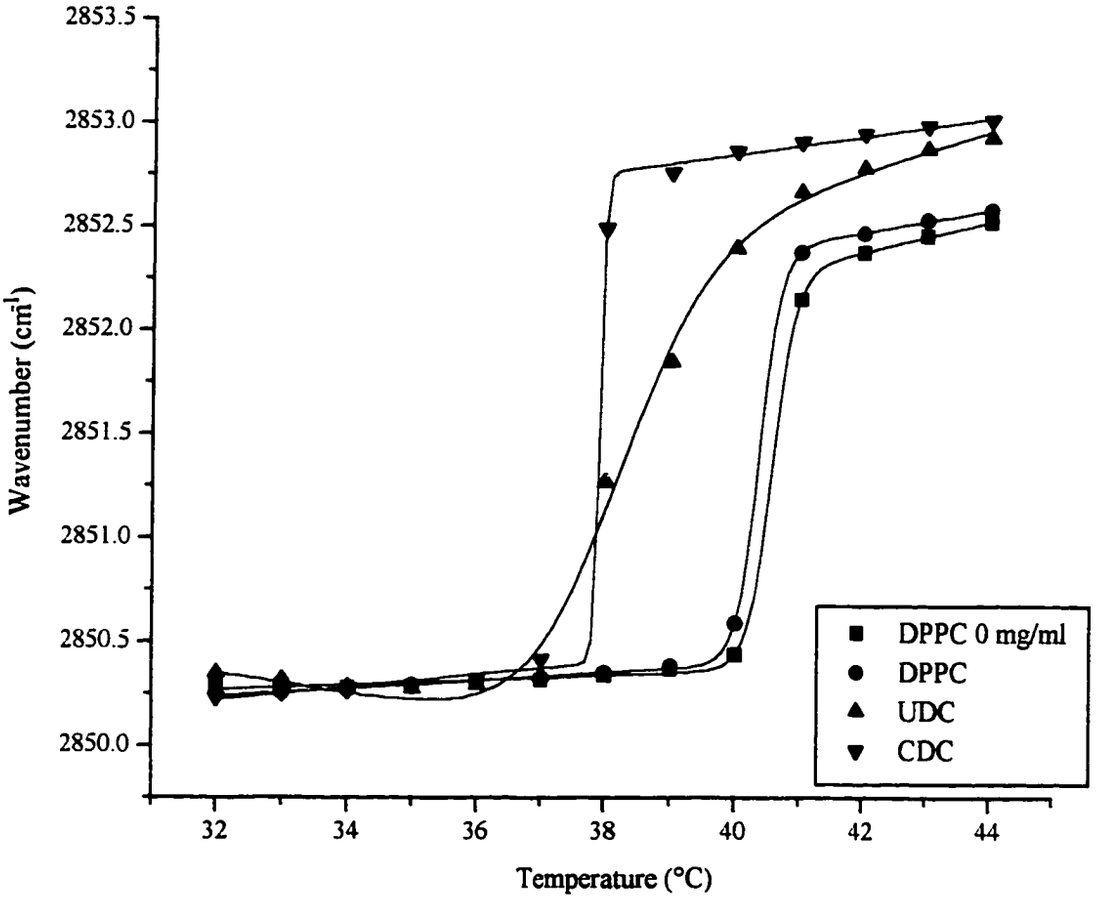


Figure 3.4: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 7)

Table 3.4: Phase Transition Parameters for Samples Hydrated with 50 mM KH_2PO_4 Solutions (Initial pH = 7)

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	55 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2850.3	2850.3	2850.2	2850.2
	44 °C*	2852.5	2852.6	2852.7	2853.1
	ΔT (°C)	0.7	0.7	0.5	0.6
	ΔT_m (°C)**		0.2	1.3	0.1
UDC	32 °C*	2850.4	2850.3	2850.2	2850.2
	44 °C*	2853.0	2852.9	2853.1	2853.2
	ΔT (°C)	3.1	2.8	0.6	0.8
	ΔT_m (°C)**	1.8	2.5	1.3	0.8
CDC	32 °C*	2850.2	2850.2	2850.3	2850.2
	44 °C*	2853.1	2853.0	2853.1	2853.2
	ΔT (°C)	2.8	0.7	1.3	1.0
	ΔT_m (°C)**	3.0	3.0	2.6	1.5

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC\ (0\ mg/ml\ d6\text{-}ETOH)} - T_{Bile\ Salt}$)

Experiments were also performed to investigate the effects which ethanol-d_6, at concentrations within the range known to induce interdigitation, has on the interactions which the bile salts have with the DPPC bilayer. DPPC samples with and without the addition of bile salt were hydrated with a solution containing 55 mg/ml ethanol-d_6.[96] The phase transition profiles for pure DPPC and DPPC samples containing either UDC or CDC hydrated with a 50 mM KH_2PO_4 solution containing 55 mg/ml ethanol-d_6 having an initial pH of 7 are given in Figure 3.5. The level of acyl chain disorder in the liquid crystalline phase increases for the sample containing UDC hydrated with a solution containing 55 mg/ml ethanol-d_6 compared to the sample hydrated with a solution containing 20 mg/ml ethanol-d_6. The increase in acyl chain disorder is indicated by the increase of 0.2 cm^{-1} in the frequency of the methylene symmetric stretching band. The level of acyl chain disorder did not change significantly for the pure DPPC or the DPPC sample containing CDC hydrated with a 55 mg/ml ethanol-d_6 containing solution when compared to similar samples hydrated with 20 mg/ml ethanol-d_6. The acyl chain order in the gel phase did not change for any of the samples. The phase transition temperatures increase for the bile salt containing samples when compared to the samples which were hydrated with a 50 mM KH_2PO_4 solution without ethanol-d_6. The phase transition temperature for the DPPC sample hydrated with a solution containing 55 mg/ml ethanol-d_6 decreases by 1.3 °C when compared to the samples which were hydrated with a 50 mM KH_2PO_4 solution containing no ethanol-d_6. The breadths of the phase transitions for all samples hydrated with a solution containing 55 mg/ml ethanol-d_6 decreased when compared to similar samples hydrated with 50 mM KH_2PO_4 solutions containing 0 mg/ml

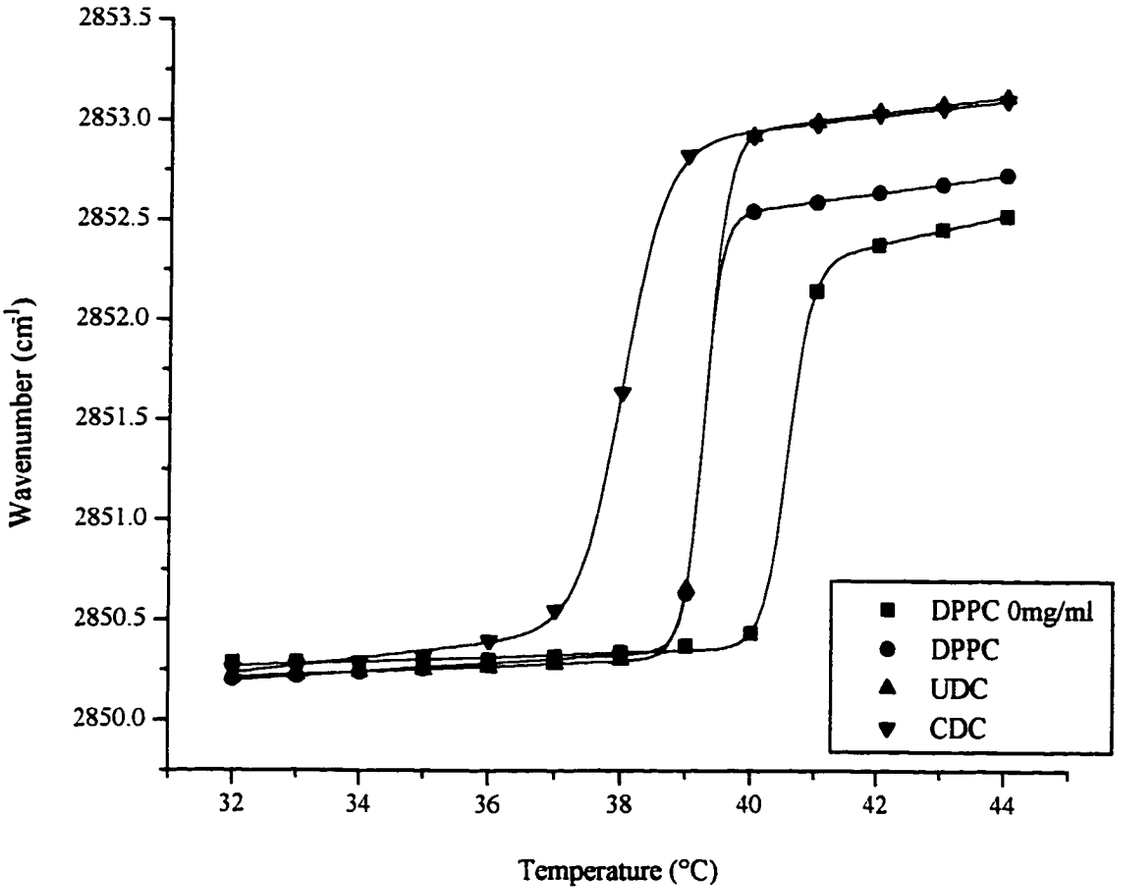


Figure 3.5: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 55 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 7)

ethanol-d_6. The methylene symmetric stretching band frequencies, ΔT_m's and breadths of the phase transitions for the samples hydrated 50 mM KH_2PO_4 solutions containing 55 mg/ml ethanol-d_6 are given in Table 3.4.

To determine if increasing the concentration of ethanol-d_6 in the hydrating solution affects the interactions which the bile salts have with the DPPC bilayer, experiments were performed using hydrating solutions containing 120 mg/ml ethanol-d_6. Phase transition profiles for samples hydrated with a 50 mM KH_2PO_4 solution having an ethanol-d_6 concentration of 120 mg/ml are given in Figure 3.6. The level of disorder of the lipid's acyl chains in the liquid crystalline phase for pure DPPC hydrated with the solution containing 120 mg/ml ethanol-d_6 increases as indicated by the 0.4 cm^{-1} increase in the frequency of the methylene symmetric stretching band when compared to the DPPC sample hydrated with a solution containing 55 mg/ml ethanol-d_6. The level of disorder in the gel phase remained the same for pure DPPC hydrated with the 50 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 in comparison to pure DPPC hydrated with a solution containing 55 mg/ml ethanol-d_6. The level of acyl chain disorder in either phase for the bile salt containing samples did not change significantly from the levels observed for the samples hydrated with 55 mg/ml ethanol-d_6. The phase transition temperatures increase for all samples (ie., the samples of pure DPPC, DPPC with 15 mole percent CDC and DPPC with 15 mole percent UDC) at this concentration of ethanol-d_6. The phase transition temperatures obtained for these samples are almost equal to the phase transition temperatures of the pure DPPC sample hydrated with a solution containing no ethanol-d_6. The phase transition parameters for these samples are presented in Table 3.4. The phase

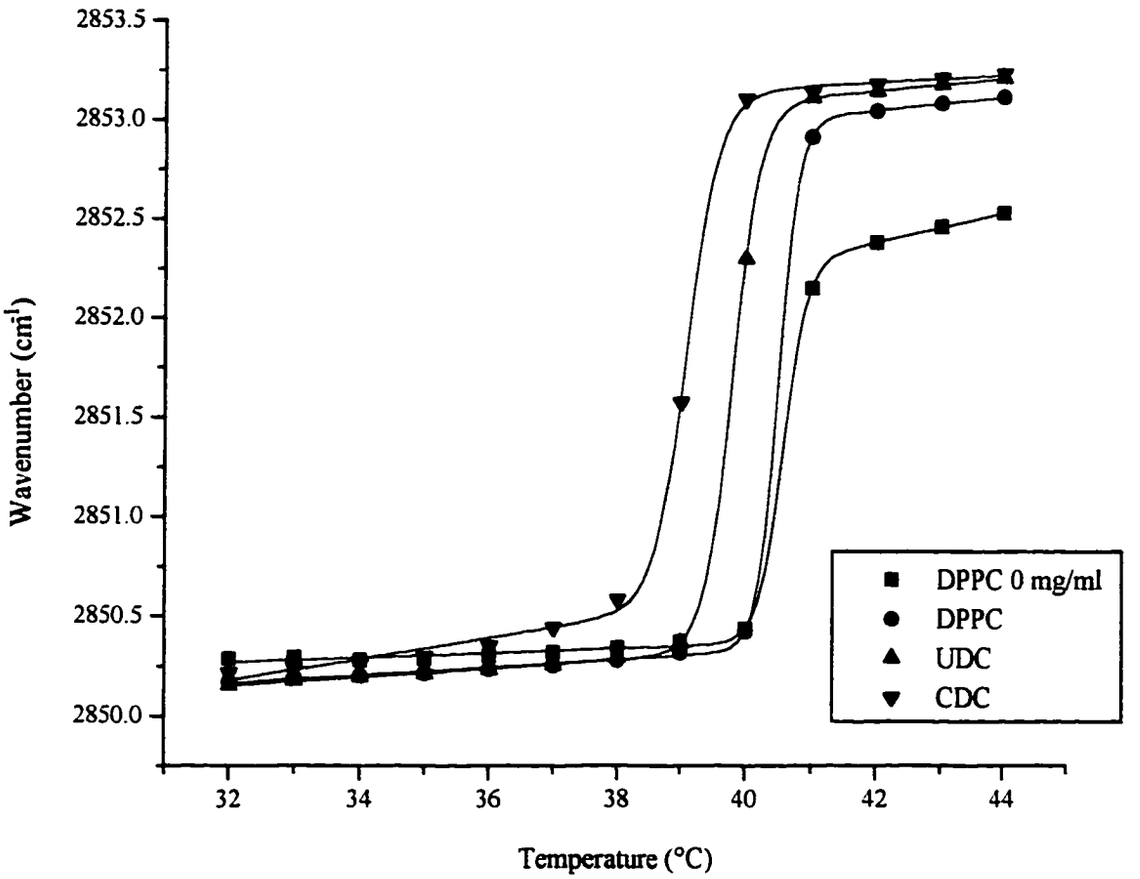


Figure 3.6: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 7)

transition temperature for the CDC containing sample was lower the UDC containing sample indicating the presence of CDC destabilizes the bilayer more than the presence of UDC under these hydrating conditions. The breadth of the phase transition for all samples hydrated with a solution containing 120 mg/ml ethanol-d_6 are less than or equal to 1 °C, indicating that the cooperativity between the lipid molecules during the phase transition is high. The phase transition parameters for the samples hydrated with a 50 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 are given in Table 3.4.

Additional experiments were performed to determine the effect which lowering the pH of the ethanol-d_6 containing hydrating solutions has on the interactions between the bile salt and the lipid assembly. Phase transition profiles for DPPC with and without the addition of either UDC or CDC hydrated with a 50 mM KH_2PO_4 solution having an ethanol-d_6 concentration of 20 mg/ml and an initial pH of 5 are given in Figure 3.7. The level of disorder of the lipid's acyl chains in the gel and the liquid crystalline phases for both the pure DPPC and the CDC containing sample hydrated with a solution containing 20 mg/ml ethanol-d_6 is the same as was observed for these bile salt containing samples hydrated with a 50 mM KH_2PO_4 solution that does not contain any ethanol-d_6. The UDC containing sample hydrated under similar conditions shows a decrease of 0.2 cm^{-1} in the frequency of the methylene symmetric stretching band in the gel phase while the frequency of this band in the liquid crystalline phase remains unchanged. Thus, the presence of ethanol-d_6 in the hydrating solution for the UDC containing sample results in a more ordered gel phase. The phase transition temperatures for the bile salt containing samples are within 0.3 °C of those obtained for similar samples hydrated with the 50 mM KH_2PO_4

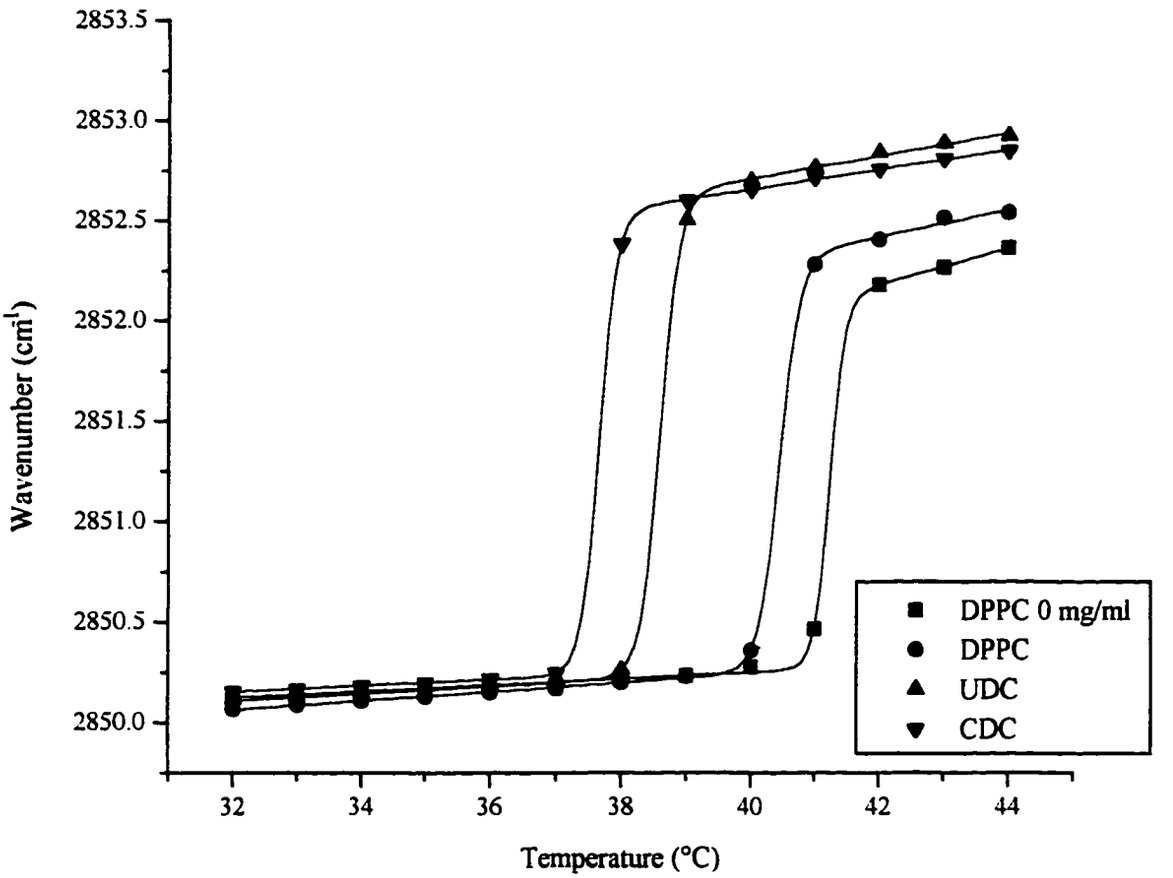


Figure 3.7: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

solution initially at a pH of 5 containing no ethanol-d_6. The phase transition temperature for the DPPC sample hydrated with the 20 mg/ml ethanol-d_6 containing solution decreases by 0.7 °C, when compared to the sample hydrated with the 50 mM KH_2PO_4 solution which does not contain any ethanol-d_6. The breadths of the phase transitions for all samples hydrated with a solution containing 20 mg/ml ethanol-d_6 are less than 1 °C. This indicates that the cooperativity of the lipid molecules during the phase transitions is high. The phase transition parameters are given in Table 3.5.

The experiments were repeated using hydrating solutions containing 55 mg/ml ethanol-d_6. The phase transition profiles for DPPC, DPPC/UDC and DPPC/CDC hydrated with a 50 mM KH_2PO_4 solution containing 55 mg/ml ethanol-d_6 having an initial pH of 5 are given in Figure 3.8. The level of disorder in the liquid crystalline phase increases for all samples hydrated with the 55 mg/ml ethanol-d_6 solution when compared to samples hydrated with the 50 mM KH_2PO_4 solution containing no added ethanol-d_6. The decrease in the level of acyl chain disorder is indicated by the increase in the frequency of the methylene symmetric stretching band. The frequency increases by 0.2 cm^{-1} for all samples. There is no significant change in the level of disorder in the gel phase for any of the samples when compared to the samples which were hydrated with solutions having 0 or 20 mg/ml ethanol-d_6. The phase transition temperatures for the DPPC samples which contain the bile salts hydrated with a solution containing 55 mg/ml ethanol-d_6 increase by at least 0.6 °C when compared to the bile salt containing samples hydrated with a 50 mM KH_2PO_4 solution containing 0 mg/ml ethanol-d_6. The phase transition temperature for the pure DPPC sample decreased by 1.8 °C when compared to the DPPC

Table 3.5: Phase Transition Parameters for Samples Hydrated with 50 mM KH_2PO_4Solutions (Initial pH = 5)

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	55 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2850.1	2850.1	2850.1	2850.0
	44 °C*	2852.4	2852.5	2852.6	2853.0
	ΔT (°C)	0.6	0.6	0.4	0.6
	ΔT_m (°C)**		0.7	1.8	-0.1
UDC	32 °C*	2850.3	2850.1	2850.2	2850.2
	44 °C*	2852.9	2852.9	2853.1	2853.1
	ΔT (°C)	1.0	0.6	0.5	0.2
	ΔT_m (°C)**	2.3	2.6	1.9	1.1
CDC	32 °C*	2850.2	2850.2	2850.1	2850.2
	44 °C*	2852.9	2852.8	2853.1	2853.2
	ΔT (°C)	0.6	0.6	0.5	0.6
	ΔT_m (°C)**	3.5	3.5	2.9	2.0

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC\ (0\ mg/ml\ d6\text{-}ETOH)} - T_{Bile\ Salt}$)

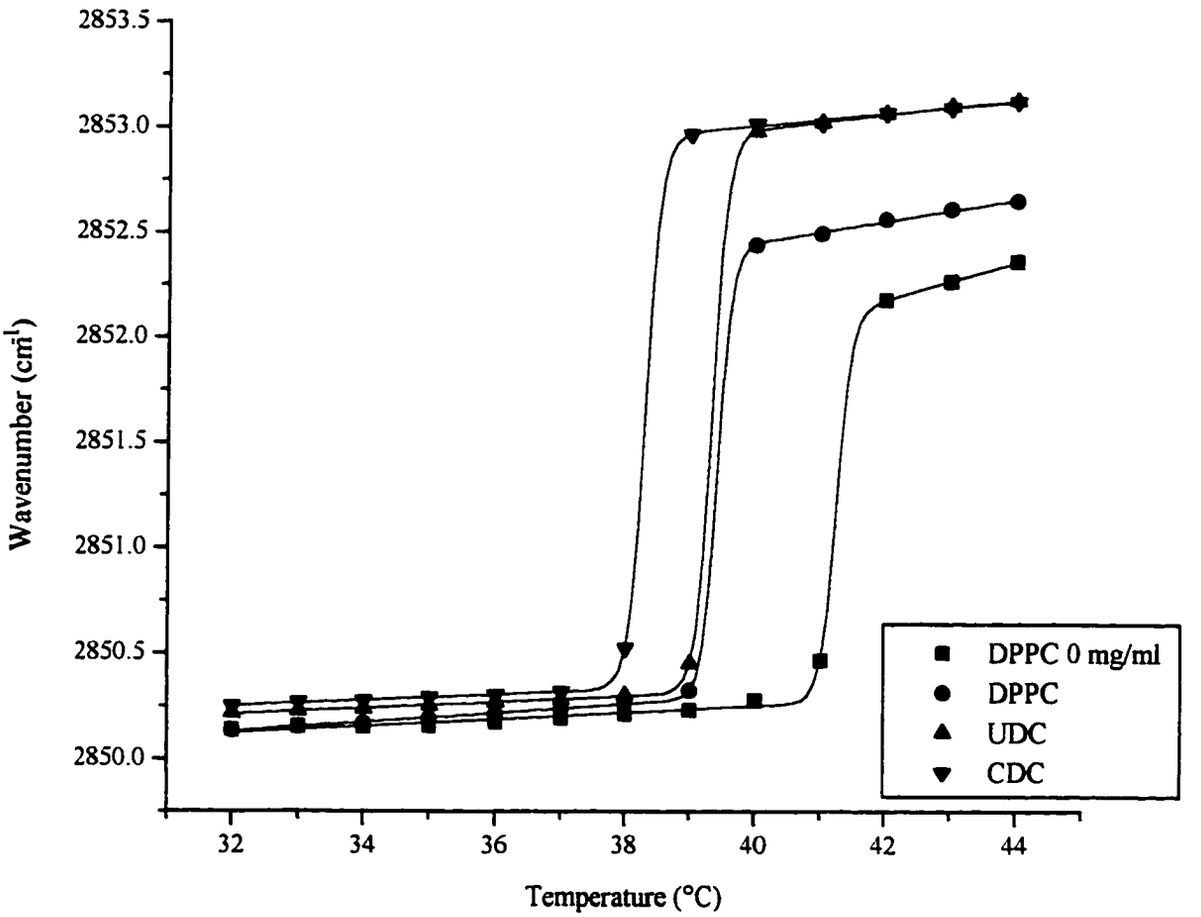


Figure 3.8: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 55 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

sample hydrated with the solution which contains only 50 mM KH_2PO_4. The breadths of the phase transitions for the DPPC samples containing the bile salt remain approximately the same as those obtained for the pure DPPC sample hydrated with solutions containing 20 mg/ml ethanol-d_6. Phase transition parameters are summarized in Table 3.5.

Experiments were also performed using samples hydrated with a 50 mM KH_2PO_4 solution having an initial pH of 5 and an ethanol-d_6 concentration of 120 mg/ml. The only changes observed for samples hydrated with this concentration of ethanol-d_6 when compared to the results obtained for hydrating solutions containing 55 mg/ml ethanol-d_6 are the level of disorder of the lipid's acyl chains for the pure DPPC sample and the phase transition temperatures for all samples. These changes can be observed in the phase transition profiles given in Figures 3.8 and 3.9. The level of disorder in the liquid crystalline phase for the pure DPPC sample hydrated with an ethanol-d_6 solution containing 120 mg/ml increases when compared to DPPC hydrated with a solution containing 55 mg/ml ethanol-d_6. The increase in the level of acyl chain disorder is indicated by the increase in the frequency of the methylene symmetric stretching band of 0.4 cm^{-1}. The level of acyl chain disorder is almost the same as for samples containing the bile salt. The level of acyl chain disorder in the gel phase did not change significantly for any of the samples when compared to samples hydrated with a solution containing 55 mg/ml ethanol-d_6. The frequencies of the methylene symmetric stretching band and the breadths of the phase transitions are given in Table 3.5. The phase transition temperatures for the DPPC samples containing either UDC or CDC increased to the point where they are now similar to the phase transition temperature of pure DPPC hydrated with a 50 mM

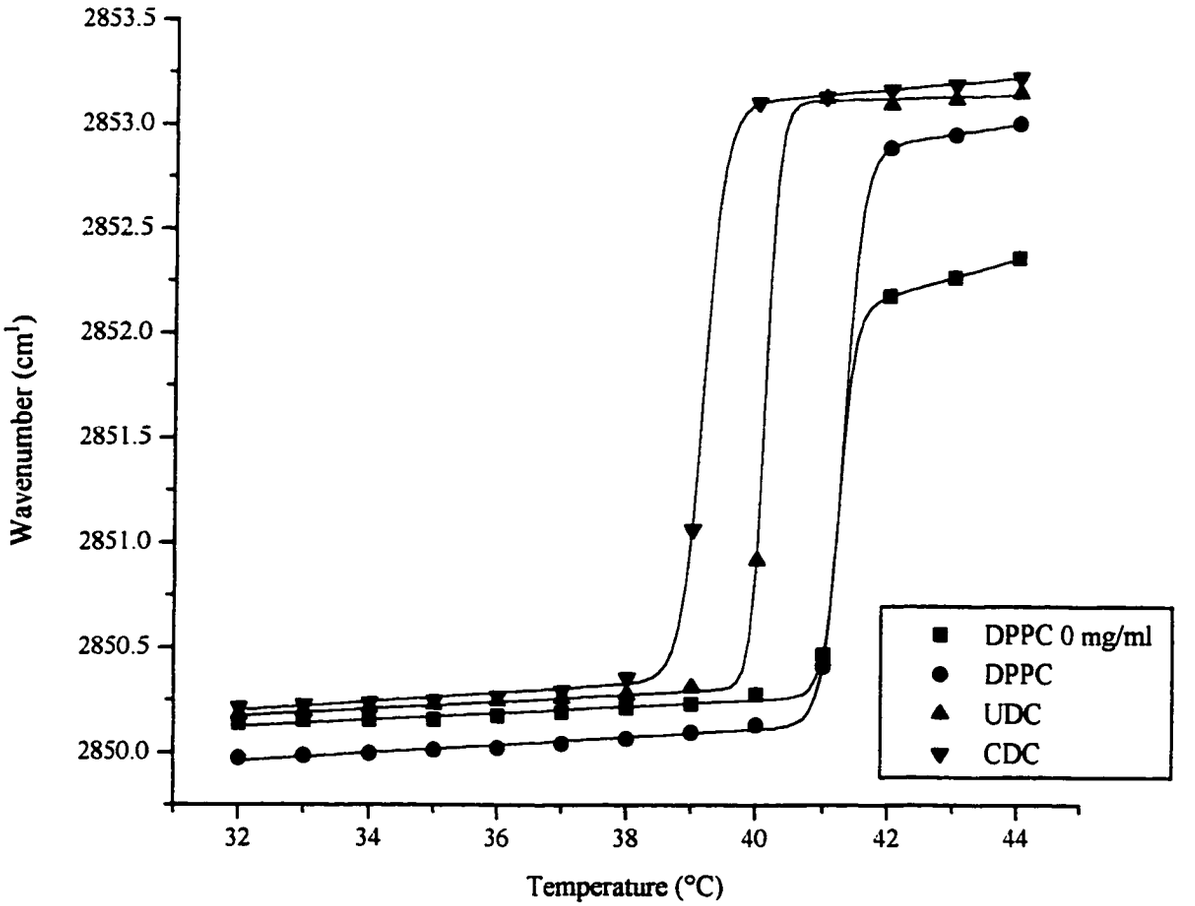


Figure 3.9: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

KH_2PO_4 solution that does not contain any ethanol-d_6. The phase transition temperature for the UDC containing sample hydrated with a solution containing 120 mg/ml ethanol-d_6 increases by 1.2 °C when compared to the UDC sample hydrated with a solution which does not contain ethanol-d_6. The phase transition temperature for the CDC sample hydrated under these conditions increased by 1.5 °C when compared to the CDC sample hydrated with a solution containing no ethanol-d_6. These data indicate that the presence of ethanol-d_6 increases the stability of the bilayer for bile salt containing samples. The breadths for all samples hydrated with a solution containing 120 mg/ml ethanol-d_6 did change from those observed for the samples hydrated with a 55 mg/ml ethanol-d_6 solution. Thus, the cooperativity between the lipid molecules during the phase transition is very high. The phase transition parameters are summarized in Table 3.5.

Experiments were conducted to evaluate the effect which the addition of ethanol in the hydrating solution has on the interactions between the bile salts and the lipid assembly for hydrating solutions having an initial pH of 8. The frequency of the methylene symmetric stretching band, ΔT_m's and the breadths of the phase transitions for DPPC samples, hydrated with a 50 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6, at an initial pH of 8, with and without the addition of the bile salts are given in Table 3.6. The phase transition profiles for these samples are given in Figure 3.10. The phase transition profiles for the bile salt containing samples hydrated with solutions which contain ethanol-d_6 lose their biphasic characteristics. A change in the level of disorder for the lipid's acyl chains in the gel phase is observed in pure DPPC and the UDC containing sample hydrated with a 20 mg/ml ethanol-d_6 containing solution when compared to samples hydrated with

Table 3.6: Phase Transition Parameters for Samples Hydrated with 50 mM KH_2PO_4 Solutions (Initial pH = 8)

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	55mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C *	2850.2	2850.0	2850.2	2850.1
	44 °C*	2852.4	2852.5	2852.7	2853.1
	ΔT (°C)	1.0	0.6	0.2	0.6
	ΔT_m (°C)**		0.4	1.0	0.2
UDC	32 °C*	2850.4	2850.2	2850.2	2850.2
	44 °C*	2852.7	2852.7	2853.0	2853.1
	ΔT (°C)	0.4[a], 5.7[b]	1.7	0.3	0.4
	ΔT_m (°C)**	5.6[a], 0.7[b]	2.3	1.6	0.6
CDC	32 °C*	2850.3	2850.2	2850.2	2850.2
	44 °C*	2852.9	2852.7	2853.0	2853.2
	ΔT (°C)	0.8[a], 4.5[b]	1.2	1.0	0.7
	ΔT_m (°C)**	5.9[a], 1.2[b]	3.1	1.7	0.5

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC\ (0\ mg/ml\ d6\text{-}ETOH)} - T_{Bile\ Salt}$)

[a] Data acquired from the first phase transition

[b] Data acquired from the second phase transition

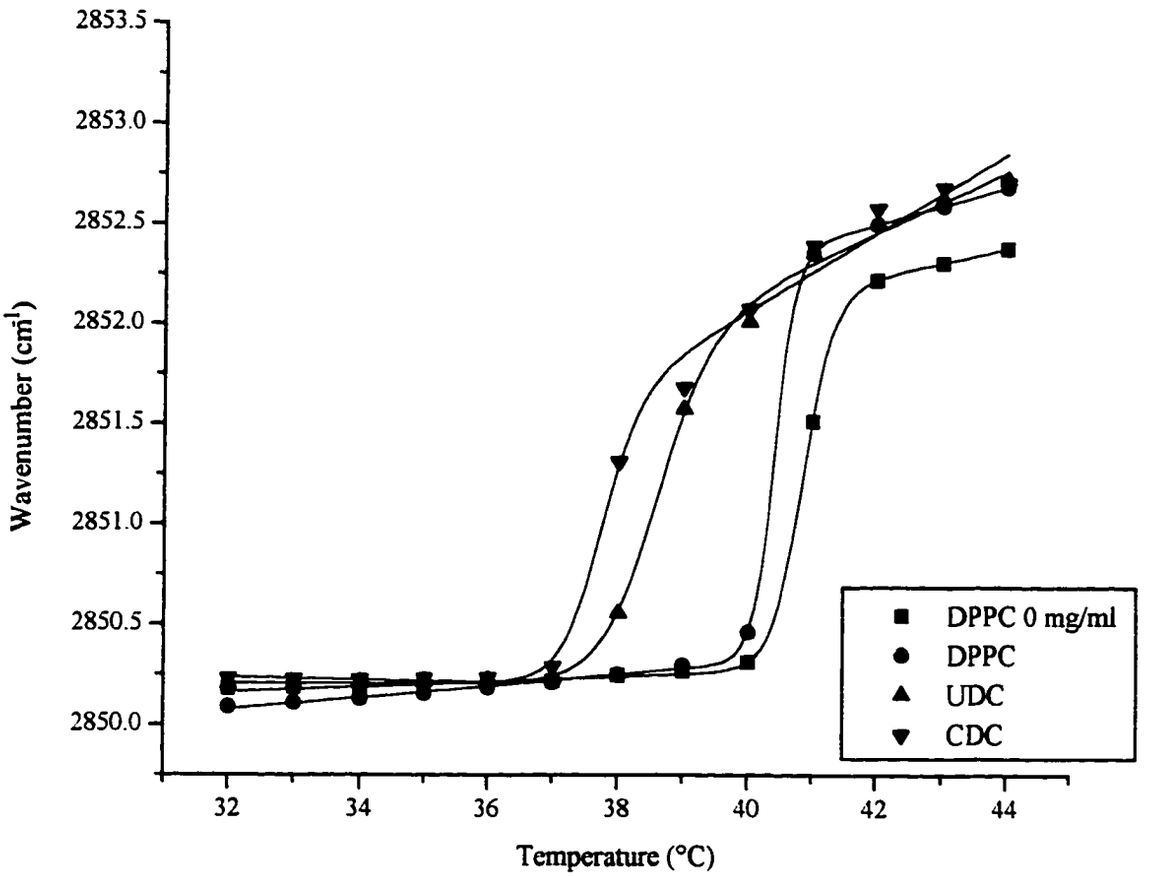


Figure 3.10: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 8)

a solution which does not contain ethanol-d_6, however, no change is observed in the level of disorder of the gel phase for the other samples. The increase in the level of acyl chain disorder is indicated by the shift in the frequency of the methylene symmetric stretching band to higher wavenumbers. The frequencies for both samples increase by 0.2 cm^{-1}. There is no change in the frequency of the methylene symmetric stretching band for the bilayer in the gel phase for the CDC containing sample hydrated with a 20 mg/ml ethanol-d_6 solution in comparison to a similar sample hydrated with a solution which does not contain ethanol-d_6. An increase in the level of disorder of the lipid's acyl chains in the liquid crystalline phase, as indicated by an increase in the frequency of the methylene symmetric stretching band, is observed for the CDC containing sample. The frequency of the methylene symmetric stretching band increases by 0.2 cm^{-1} for the CDC containing sample when compared to similar samples hydrated with a 50 mM KH_2PO_4 solution containing no ethanol. No change is observed in the level of acyl chain disorder in the liquid crystalline phase for pure DPPC or the sample containing UDC hydrated with a 20 mg/ml ethanol-d_6 solution when compared to similar samples hydrated with a solution which does not contain ethanol-d_6. The phase transition temperatures for the bile salt containing samples are lower than those observed for the high temperature phase transition of similar samples hydrated with a solution containing no ethanol-d_6. The phase transition temperature of DPPC decreases by 0.4 °C when compared to pure DPPC hydrated with a solution containing no ethanol-d_6. The breadths of the phase transitions for the UDC and CDC containing samples are lower than those obtained for the high temperature transitions of similar samples hydrated with a solution which does not contain

ethanol-d_6. The phase transition parameters are given in Table 3.6.

Additional experiments were performed to evaluate the effect which increasing the ethanol concentration in the hydrating solution has on the interactions which occur between the bile salt and the lipid assembly. When the ethanol concentration in the hydrating solution is increased to 55 mg/ml ethanol-d_6, an increase in the level of disorder of the lipid's acyl chains in the liquid crystalline phase for all bile salt containing (DPPC containing UDC and DPPC containing CDC) samples is observed. The phase transition profiles for DPPC, DPPC containing UDC and DPPC containing CDC hydrated with a 55 mg/ml ethanol-d_6 solution are given in Figure 3.11. The level of disorder in the gel phase remains unchanged for the bile salt containing samples when compared to similar samples hydrated with a solution containing 20 mg/ml ethanol-d_6. The frequency of the methylene symmetric stretching band for pure DPPC hydrated with 20 mg/ml ethanol-d_6 is 0.2 cm^{-1} lower than that observed for pure DPPC hydrated with a solution containing no ethanol-d_6. The decrease in the frequency of the methylene symmetric stretching band indicates that the level of acyl chain disorder has decreased, ie., the bilayer is more rigid. The level of acyl chain disorder in the liquid crystalline phase for all sample hydrated with a 55 mg/ml ethanol-d_6 solution increases when compared to similar samples hydrated with a 20 mg/ml ethanol-d_6 solution. The increase in the level of acyl chain disorder is indicated by the increase in the frequency of the methylene symmetric stretching band. The frequencies increase by 0.2 cm^{-1} for pure DPPC and 0.3 cm^{-1} for the two bile salt containing samples. The phase transition temperatures increase for the bile salt containing samples when hydrated under these conditions compared to samples hydrated with a

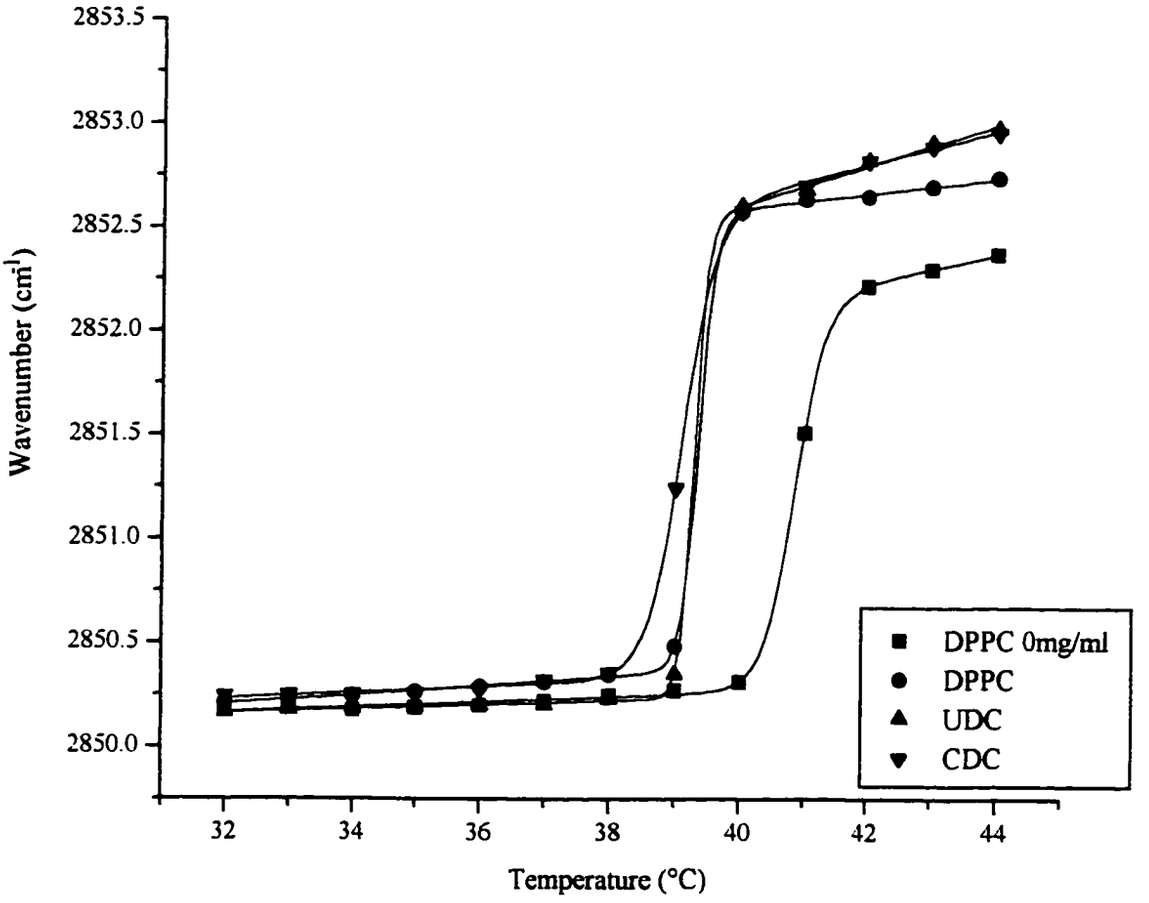


Figure 3.11: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 55 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 8)

solution containing 20 mg/ml ethanol-d_6. The pure DPPC sample which was hydrated with the 55 mg/ml ethanol containing solution shows a decrease in the phase transition temperature when compared to the pure DPPC samples hydrated with solutions containing 0 or 20 mg/ml ethanol-d_6. The phase transition temperature decreases by 1 °C from that obtained for the pure DPPC sample hydrated with the solution which does not contain ethanol. The breadths of the phase transitions decrease for all samples hydrated with a solution containing 55 mg/ml ethanol-d_6. Thus, the cooperativity between the lipid molecules during the phase transition increases for these samples when compared to the samples hydrated with 50 mM KH_2PO_4 solutions having an ethanol-d_6 concentration of 20 mg/ml. The frequencies of the methylene symmetric stretching band, the breadths of the phase transitions and the ΔT_m's are summarized in Table 3.6.

Experiments were also performed using DPPC samples, with and without bile salt, hydrated with 50 mM KH_2PO_4 solutions containing 120 mg/ml ethanol-d_6 to determine the effects which higher concentrations of ethanol-d_6 have on the bile salt/lipid interactions. The phase transition profiles for DPPC, DPPC containing UDC and DPPC containing CDC hydrated with a solution having an ethanol-d_6 concentration of 120 mg/ml are given in Figure 3.12 The level of acyl chain disorder in the liquid crystalline phase increases for all samples, except the UDC containing sample, hydrated with a 50 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 compared to similar samples hydrated with a 50 mM KH_2PO_4 solution containing 55 mg/ml ethanol-d_6. The increase in the level of acyl chain disorder is indicated by the increase in the frequency of the methylene symmetric stretching band by 0.4 cm^{-1}, for the DPPC sample and 0.2 cm^{-1} for the CDC containing

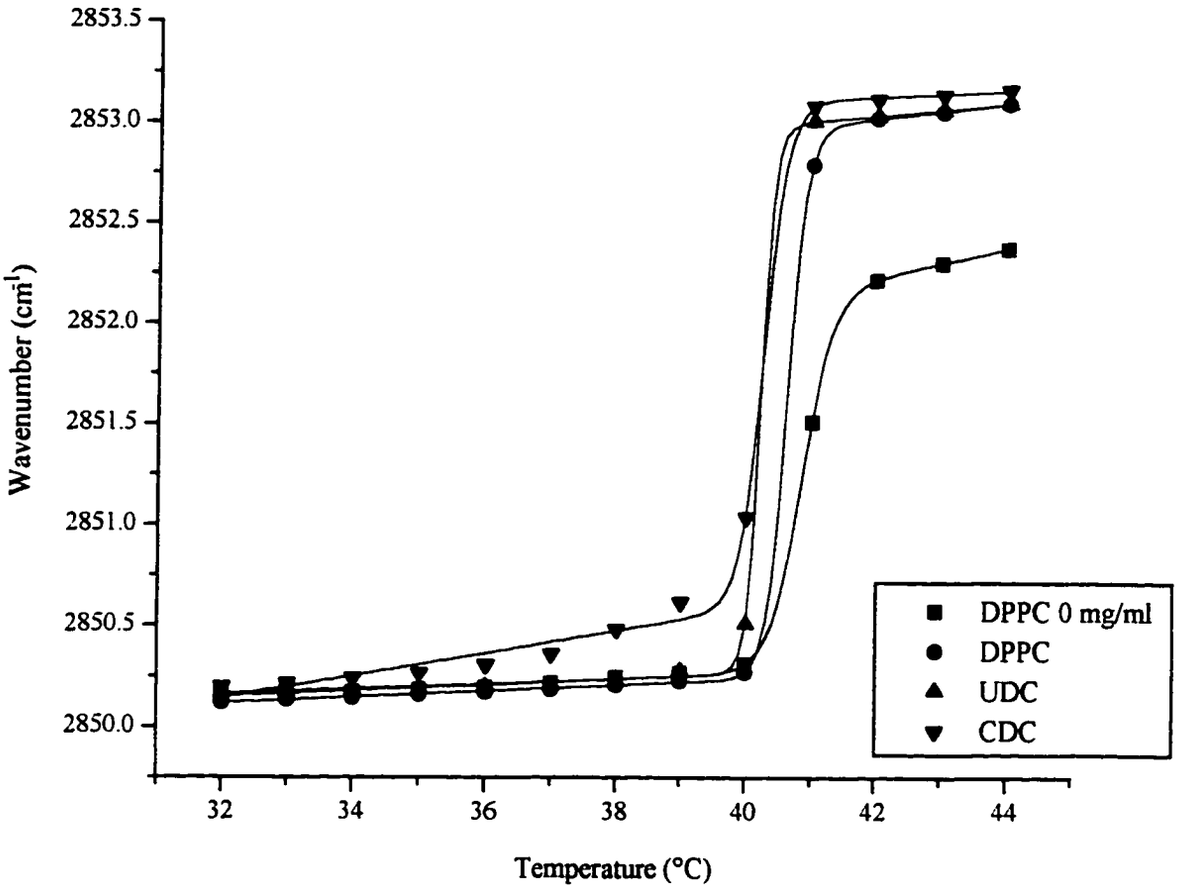


Figure 3.12: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 8)

sample that are hydrated with a solution of 50 mM KH_2PO_4 having an initial pH of 8 and an ethanol-d_6 concentration of 120 mg/ml. There is no significant change in the order parameters for any of these samples in the gel phase when compared to samples hydrated with a 50 mM KH_2PO_4 solution containing 55 mg/ml ethanol-d_6. The phase transition temperatures also increase for all samples hydrated with a solution containing 120 mg/ml ethanol-d_6. The breadths of the phase transitions for all samples hydrated with a 120 mg/ml ethanol-d_6 solution remain approximately the same as those obtained for similar samples hydrated with a solution containing 55 mg/ml ethanol-d_6. The phase transition parameters are presented in Table 3.6.

3.3.4 Effect of Adding Ethanol-d_6 to Hydrating Solutions Having a KH_2PO_4 Concentration of 200 mM

Experiments were performed using 200 mM KH_2PO_4 hydrating solutions containing various concentrations of ethanol-d_6. These experiments were performed to determine if the addition of ethanol-d_6 to the hydrating solution affects the interactions between the bile salt and the DPPC bilayer. Phase transition profiles for DPPC, with and without the addition of bile salt, hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 having an initial pH of 7 are given in Figure 3.13. The results show that the addition of low concentrations of ethanol-d_6, 20 mg/ml to the hydrating solution, for samples containing DPPC does not affect the level of disorder of the lipid's acyl chains in the gel phase. The frequency of the methylene symmetric stretch for the DPPC sample, however, increases, by 0.2 cm^{-1}, in the liquid crystalline phase when compared to a DPPC

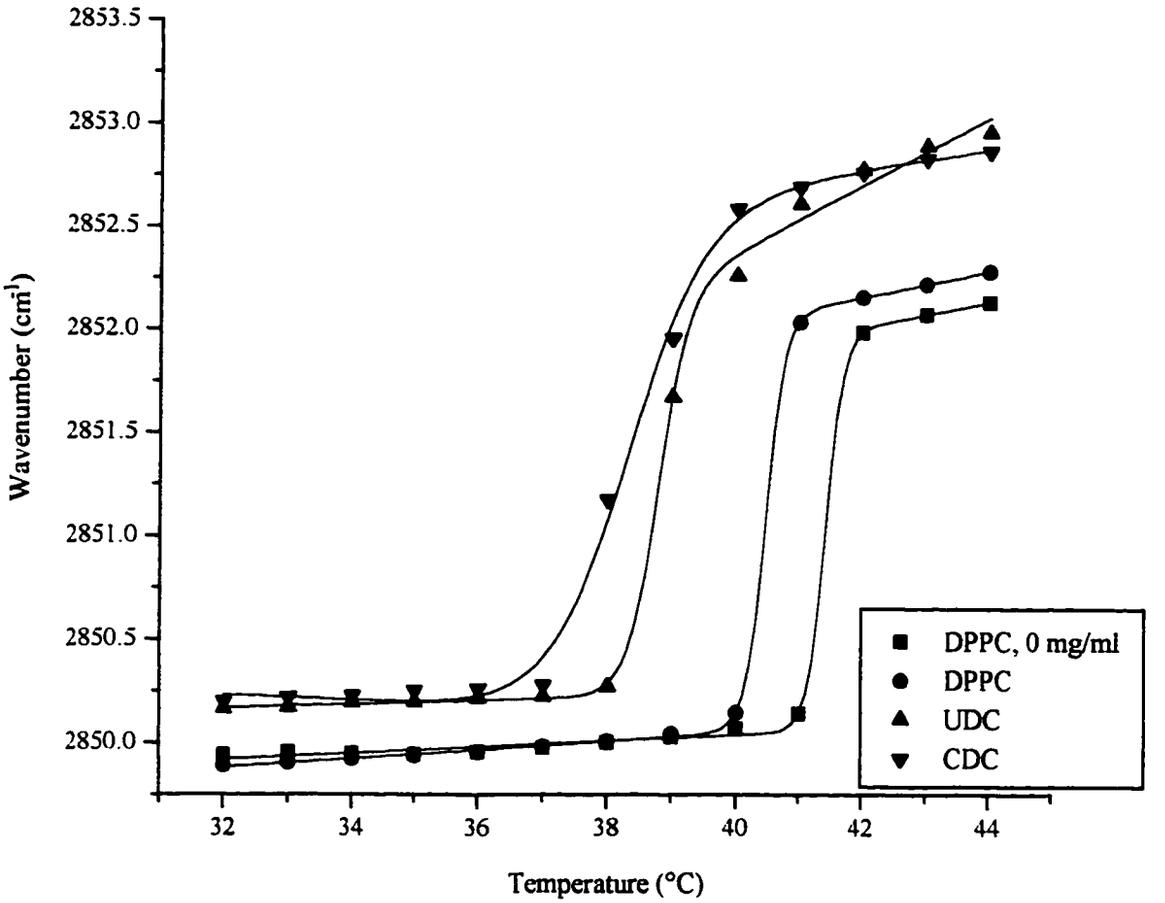


Figure 3.13: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/200 mM KH_2PO_4 (Initial pH = 7)

sample which was hydrated with a 200 mM KH_2PO_4 solution which does not contain any ethanol. Thus, the addition of this concentration of ethanol-d_6 results in an increase in the level of acyl chain disorder in the liquid crystalline phase. DPPC samples containing either UDC or CDC, at the fifteen mole percent level, do not show significant change in the level of acyl chain disorder in the gel phase when compared to samples hydrated with solutions containing no ethanol-d_6. The UDC containing sample does, however, show an increase in the level of acyl chain disorder in the gel phase when hydrated with a solution containing 20 mg/ml ethanol-d_6 in comparison to a similar sample hydrated with a solution which does not contain ethanol-d_6. The phase transition temperatures decrease for all samples. The T_m for the UDC and CDC containing samples decrease by 0.2 °C when compared to the UDC and CDC containing samples hydrated with a 200 mM KH_2PO_4 solution, at an initial pH of 7, containing no ethanol-d_6. The breadths of the phase transition profiles for the bile salt containing samples decrease with the addition of low ethanol-d_6 concentrations when compared to the bile salt containing samples hydrated with a solution containing no ethanol-d_6. This decrease in breadth indicates an increase in the cooperativity between the lipid molecules during the phase transition. The phase transition parameters are summarized in Table 3.1.

Studies were also conducted using 200 mM KH_2PO_4 hydrating solutions containing 120 mg/ml ethanol-d_6. This concentration of ethanol (2.3 M) is well above the level necessary to cause the interdigitation of the pure lipid's acyl chains. The frequencies of the lipid's methylene symmetric stretching band, ΔT_m's and ΔT's for the samples hydrated with a solution containing 120 mg/ml ethanol-d_6 are given in Table 3.1 and the phase transition

profiles for these samples are given in Figure 3.14. The level of disorder of the lipid's acyl chains in the liquid crystalline phase for the DPPC sample hydrated with the 200 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 increases as indicated by the increase, of 0.9 cm^{-1}, in the frequency of the methylene symmetric stretching band when compared to pure DPPC hydrated with a 200 mM KH_2PO_4 solution, having an initial pH of 7, containing 0 mg/ml ethanol-d_6. The frequency for the DPPC sample hydrated with a 200 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 is approximately at the same level as the CDC and UDC containing samples hydrated with the same solution. The frequency of the methylene symmetric stretch of the lipid's acyl chains in the gel phase decreased for the UDC and CDC containing samples, by 0.3 cm^{-1} and by 0.2 cm^{-1}, respectively, compared to similar samples which were hydrated with a solution which did not contain any ethanol-d_6. The phase transition temperatures for the bile salt containing samples increased compared to the samples hydrated with solutions containing lower ethanol-d_6 concentrations and are within 1.0 °C and 1.1 °C, for the UDC and CDC containing samples, respectively, of the phase transition temperature of the pure DPPC sample hydrated with a solution which does not contain ethanol-d_6. The breadths of the phase transition decreased, from 6.2 ° to 0.6 °C and from 5.3 °C to 0.6 °C for the samples which contain UDC or CDC, respectively, when compared to those samples hydrated with a 200 mM KH_2PO_4 solution (initial pH of 7) which contains no ethanol-d_6.

Experiments were repeated using hydrating solutions having an initial pH of 5. Phase transition profiles for DPPC, DPPC containing 15 mole percent UDC and DPPC containing 15 mole percent CDC hydrated with a 200 mM KH_2PO_4 solution containing 20

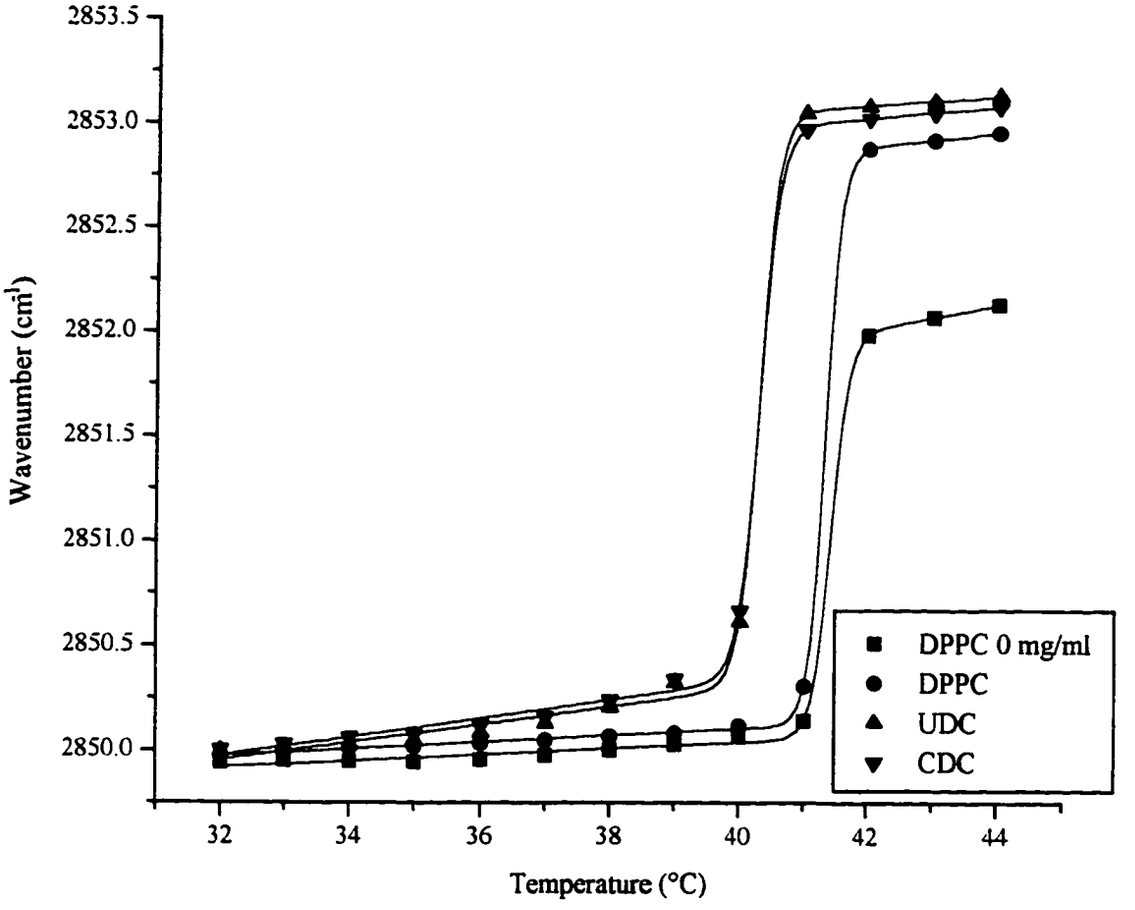


Figure 3.14: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4Solution (Initial pH = 7)

mg/ml ethanol-d_6 are given in Figure 3.15. The level of acyl chain disorder in the gel phase for all of the samples hydrated with a 200 mM KH_2PO_4 solution, having an initial pH of 5, containing 20 mg/ml ethanol-d_6 does not differ significantly from the samples hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 5 with no added ethanol-d_6. The level of acyl chain disorder in the liquid crystalline phase, however, increases for a pure DPPC sample hydrated with the solution containing 20 mg/ml ethanol-d_6 compared to a DPPC sample hydrated with a 200 mM KH_2PO_4 solution containing no added ethanol-d_6. Thus, the addition of ethanol-d_6 produces a more fluid liquid crystalline phase for pure DPPC. The bile salt containing samples show no significant change in the frequency of the methylene symmetric stretching mode in the liquid crystalline phase when compared to similar samples hydrated with a 200 mM KH_2PO_4 solution that contains no ethanol-d_6. The phase transition temperature of the UDC containing sample is 1.0 °C higher than the phase transition temperature for a similar sample hydrated with a 200 mM KH_2PO_4 solution containing no ethanol-d_6. The phase transition temperature of the CDC containing sample shows an increase of 0.5 °C from the phase transition temperature for the sample hydrated with a 200 mM KH_2PO_4 solution containing no ethanol-d_6. The phase transition temperature for the DPPC sample hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6, however, decreases by 0.9 °C compared to the sample hydrated with a solution having an ethanol-d_6 concentration of 0 mg/ml. The breadths of the phase transitions obtained for samples hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 change slightly from those of similar samples hydrated with a solution which does not contain any ethanol-d_6. The breadth of the phase transition

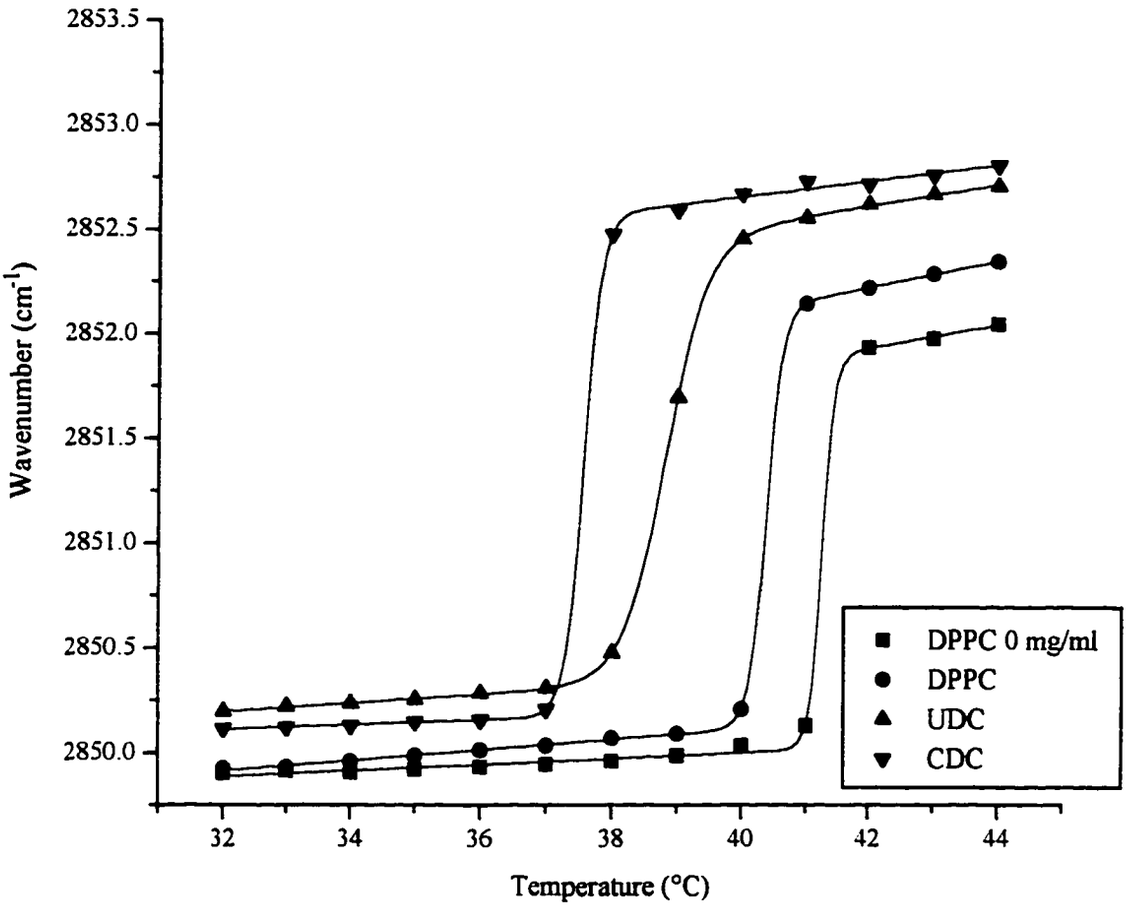


Figure 3.15: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 5)

decreases from 1.8 °C for the CDC containing sample hydrated with a 200 mM KH_2PO_4 solution containing no ethanol-d_6 to 0.6 °C for the sample hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 and from 1.0 °C for the UDC containing sample hydrated with a 200 mM KH_2PO_4 solution containing no ethanol-d_6 to 1.3 °C for the sample hydrated with a 200 mM KH_2PO_4 solution having an ethanol-d_6 concentration of 20 mg/ml. The sharp phase transition profiles for all samples indicate that there is a high degree of cooperativity between the lipid molecules during the phase transition. The breadths of the phase transitions, the frequencies of the methylene symmetric stretching band and the ΔT_m's for these samples are given in Table 3.2

To determine the effects which adding ethanol-d_6 at higher concentrations to the 200 mM KH_2PO_4 solution has on the bile salt/lipid interactions, experiments were performed using solutions having an ethanol-d_6 concentration of 120 mg/ml. The breadths of the phase transitions for the bile salt containing samples decrease as shown in Figure 3.16. These data indicate an increase in the cooperativity between the lipid molecules during the phase transition. The level of acyl chain disorder in the gel phase is not significantly different for any of the samples when compared to samples hydrated with the 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6. However, the level of acyl chain disorder in the liquid crystalline phase for all samples hydrated with a 120 mg/ml ethanol-d_6 containing solution increases from the level observed for samples hydrated with solutions containing 0 or 20 mg/ml ethanol-d_6. The level of acyl chain disorder in the liquid crystalline phase for all of the samples hydrated with 120 mg/ml ethanol-d_6 solutions are similar. The phase transition temperatures for the bile salt samples hydrated with a

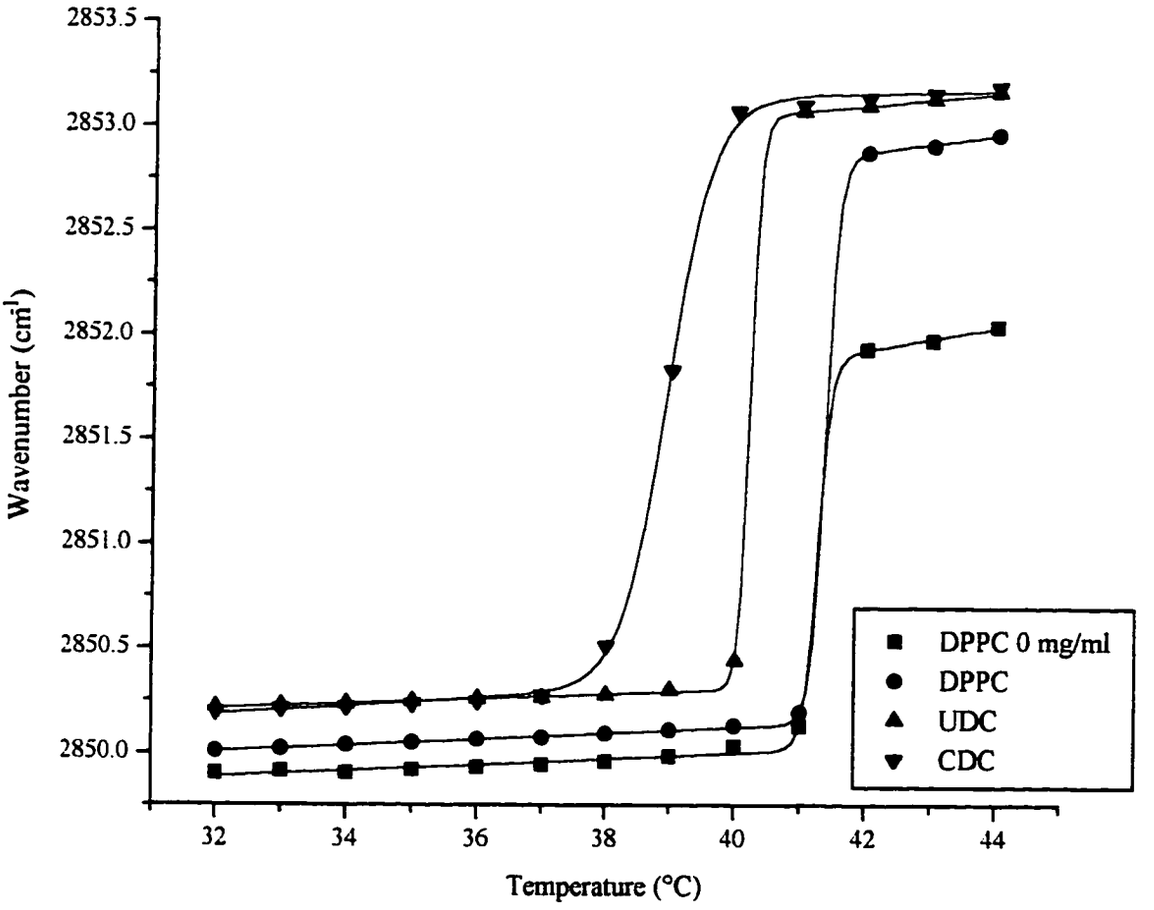


Figure 3.16: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 5)

solution containing 120 mg/ml ethanol-d_6 have increased when compared to the bile salt containing samples hydrated with a solution containing 20 mg/ml ethanol-d_6 and are now closer to the phase transition temperature of the pure DPPC sample hydrated with a similar solution containing no ethanol-d_6. The CDC containing sample has a lower phase transition temperature, 38.9 °C, than the UDC containing sample which indicates that CDC epimer destabilizes the bilayer more under these hydrating conditions. The phase transition parameters are given in Table 3.2.

Studies were conducted using hydrating solutions with a KH_2PO_4 concentration of 200 mM, at an initial pH of 8, to determine if the addition of ethanol-d_6 to the hydrating solution affects the interactions which the bile salts have with the DPPC bilayer hydrated with solutions having higher initial pH's. It should be noted the phase transition profiles, presented in Figure 3.17, for samples containing either bile salt when hydrated with any ethanol-d_6 containing solution show no indication of being biphasic. Thus, it appears that the addition of ethanol to the hydrating solution produces a more uniform affect of the bile salt on the lipid bilayer. The level of disorder of the lipid's acyl chains in the liquid crystalline phase increases for the CDC containing sample hydrated with a 200 mM KH_2PO_4 solution which contains 20 mg/ml ethanol-d_6 when compared to similar samples hydrated with a 200 mM KH_2PO_4 solution containing 0 mg/ml ethanol-d_6. The increase in the level of acyl chain disorder is indicated by the increase in the frequency of the methylene symmetric stretching band of the lipid. The increase in the frequency of the methylene symmetric stretching bands of the lipid's acyl chains for the CDC containing sample is 0.2 cm^{-1}. No significant change in the level of acyl chain disorder in the liquid

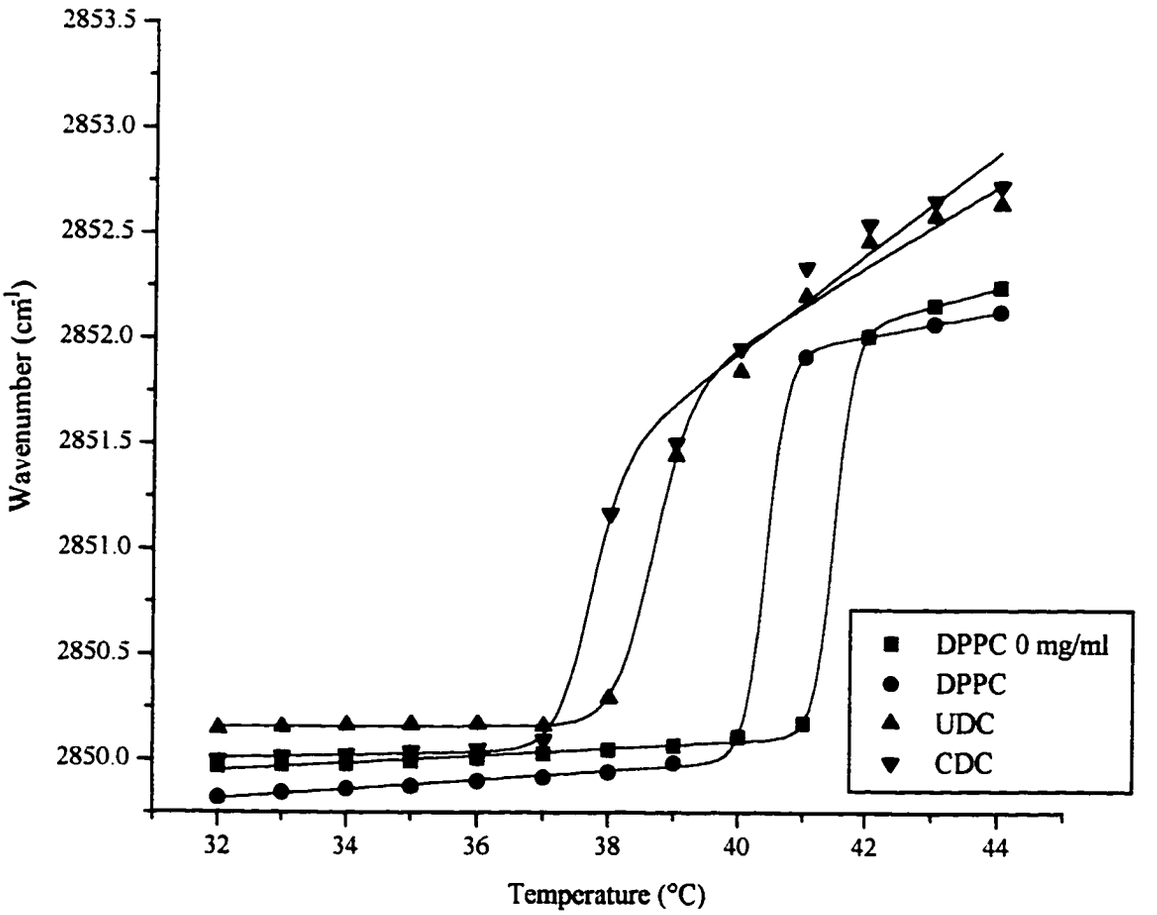


Figure 3.17: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 8)

crystalline phase is observed between the UDC containing sample hydrated with a solution which contains 20 mg/ml ethanol-d_6 and a solution containing no ethanol-d_6. The frequency of the methylene symmetric stretching mode decreases by 0.2 cm^{-1} in the gel phase for the DPPC sample hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 compared to the pure DPPC sample hydrated with a solution which contains no ethanol-d_6. The decrease in the band frequency indicates a decrease in the level of acyl chain disorder. The level of acyl chain disorder in the gel phase for the sample which contains UDC hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 decreases from that obtained for the UDC containing sample hydrated with a solution which does not contain any ethanol-d_6. The change in the level of acyl chain disorder is indicated by a shift in the frequency of the methylene symmetric stretch to lower wavenumbers. The phase transition temperature for the pure DPPC sample hydrated with this ethanol-d_6 containing solution is 1.1 °C lower than the phase transition temperature of pure DPPC hydrated with a 200 mM KH_2PO_4 solution with no added ethanol-d_6. The breadths of the phase transitions for the bile salt containing samples hydrated with the 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 decrease compared to the breadths of the higher temperature phase transitions for the bile salt containing samples hydrated with a 200 mM KH_2PO_4 solution containing no ethanol-d_6. These data once again indicate that there is an increase in the cooperativity between the lipid molecules during the phase transition when ethanol-d_6 is present in the hydrating solution. The phase transition parameters for these samples are summarized in Table 3.3.

Increasing the concentration of ethanol-d_6 in the hydrating solution, results in an

increase in the level of acyl chain disorder in the liquid crystalline phase for all samples hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 8 and an ethanol-d_6 concentration of 120 mg/ml when compared to samples hydrated with a solution having an ethanol-d_6 concentration of 20 mg/ml. This effect can be observed by comparing the phase transition profiles given in Figures 3.17 and 3.18. The level of acyl chain disorder in the gel phase increases for the pure DPPC sample while the bile salt containing samples show no significant changes in the level of acyl chain disorder in this phase compared to similar sample hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6. The phase transition temperatures for the bile salt containing samples are closer to the phase transition temperature of pure DPPC hydrated with the same solution. The breadths of the phase transitions decrease for all samples when compared to the samples hydrated with a solution which contained 20 mg/ml ethanol-d_6. The phase transition parameters for these samples are presented in Table 3.3.

The effects which the bile salts have on the DPPC bilayer hydrated with a 50 mM KH_2PO_4 solutions containing various concentrations of ethanol-d_6 are similar to those observed for samples hydrated with a 200 mM KH_2PO_4 solutions containing similar concentrations of ethanol-d_6. All trends noted in the phase transition data are similar for both KH_2PO_4 concentrations, which suggests that the pH's of the resulting samples are similar for the two KH_2PO_4 concentrations studied.

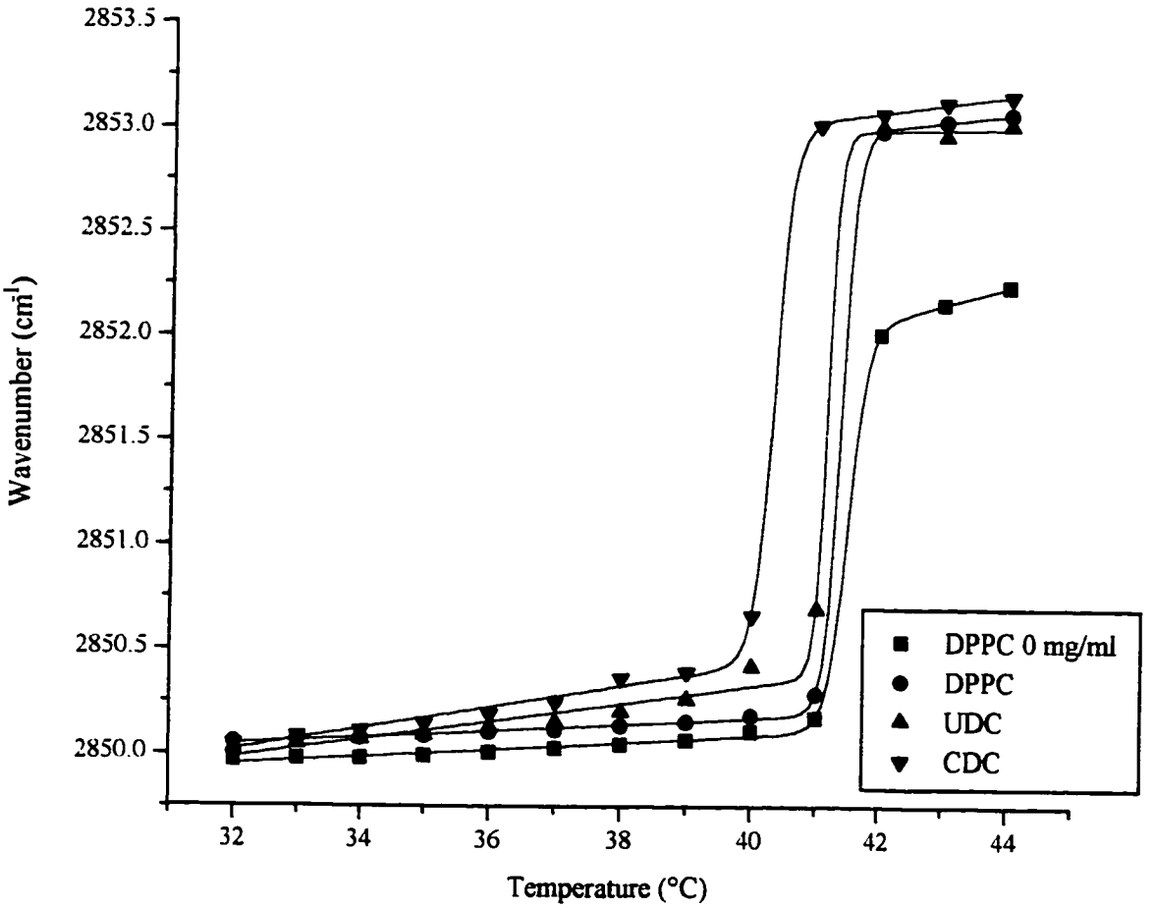


Figure 3.18: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 8)

3.3.5 Experiments to Determine the Effect which the Presence of Ethanol-d_6 in the Hydrating Solution (Initial pH of 5) has on the Bile Salt/Lipid Interactions: Raman Experiments

Raman spectra were acquired for samples of pure DPPC and DPPC with 15 mole percent UDC hydrated with 50 mM KH_2PO_4 solutions having ethanol-d_6 concentrations of 20 and 120 mg/ml and an initial pH of 5. Spectra were not corrected for the spectral contribution of the bile salt. Due to the limited time available for acquiring data, data were obtained only at temperatures of 25, 32, 44 and 48 °C. Full gel to liquid crystalline phase transition profiles were not obtained.

The I_{2850}/I_{2880} intensity ratio provides information regarding the interchain disorder of the lipid acyl chains while the I_{2935}/I_{2880} intensity ratio is indicative of both the inter- and intrachain order/disorder characteristics. That is, the I_{2935}/I_{2880} intensity ratio yields information regarding both the lateral order of the hydrocarbon chains and changes in the relative number of gauche conformers in the lipid's acyl chains.

Plots of the I_{2850}/I_{2880} and I_{2935}/I_{2880} intensity ratios versus temperature for pure DPPC hydrated with solutions containing 20 and 120 mg/ml ethanol-d_6 are given in Figures 3.19 and 3.20. The I_{2850}/I_{2880} intensity ratio of pure DPPC obtained for the lower temperatures was smaller for the sample hydrated with a solution containing 120 mg/ml ethanol-d_6 when compared to a similar sample hydrated with a solution containing 20 mg/ml ethanol-d_6. At higher temperatures, the opposite phenomenon occurs. That is, the I_{2850}/I_{2880} intensity ratio for pure DPPC hydrated with a solution containing 20 mg/ml ethanol-d_6 is lower than the intensity ratio obtained for pure DPPC hydrated with a

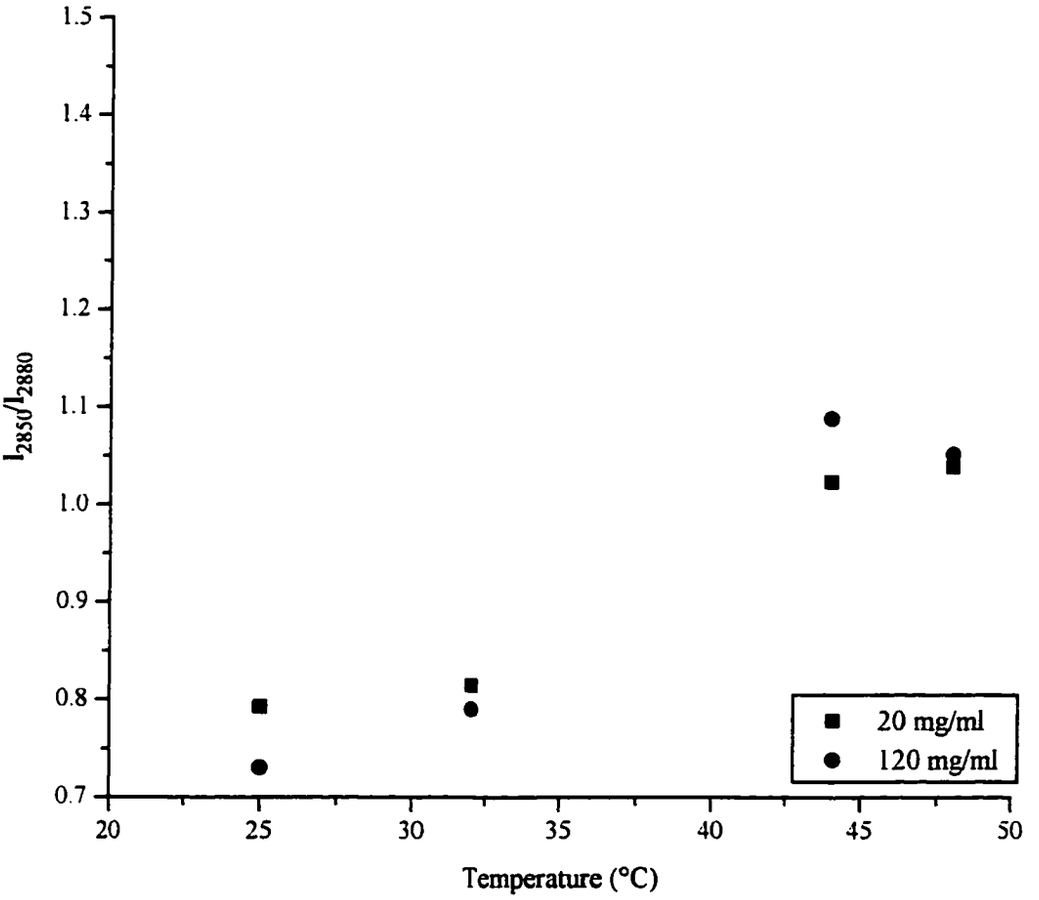


Figure 3.19: Plot of the Ratio of the Intensities at 2850 and 2880 cm^{-1} (I_{2850}/I_{2880}) versus Temperature for pure DPPC Samples Hydrated with an Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

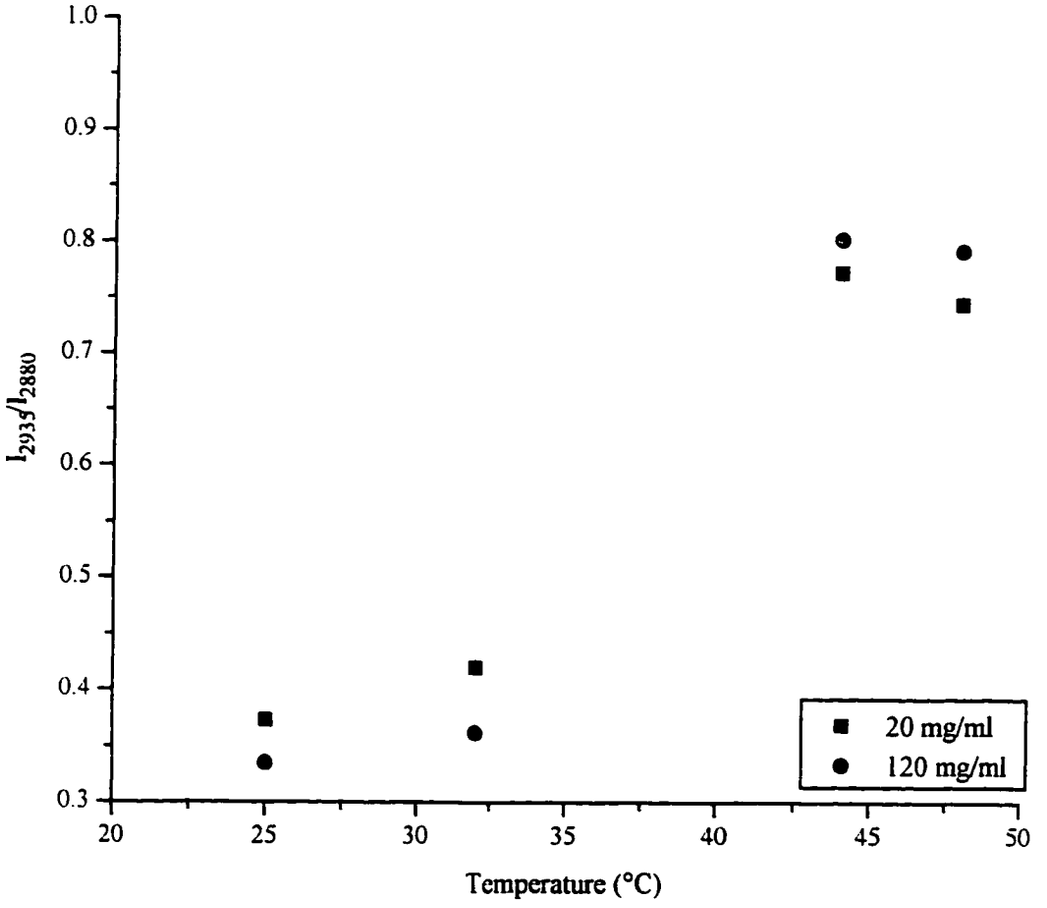


Figure 3.20: Plot of the Ratio of the Intensities at 2935 and 2880 cm^{-1} ((I_{2935}/I_{2880}) versus Temperature for pure DPPC Samples Hydrated with an Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

solution which contains 120 mg/ml ethanol-d_6. These data indicate that at 25 and 32 °C (gel phase), pure DPPC hydrated with a 50 mM KH_2PO_4 solution (initial pH = 5) containing 120 mg/ml ethanol-d_6 is more ordered than pure DPPC hydrated with a 50 mM KH_2PO_4 solution (initial pH = 5) containing 20 mg/ml ethanol-d_6. Pure DPPC hydrated with a 50 mM KH_2PO_4 solution (initial pH = 5) containing 20 mg/ml ethanol-d_6 is more ordered, at 44 and 48 °C (liquid crystalline phase), than pure DPPC hydrated with a 50 mM KH_2PO_4 solution (initial pH = 5) containing 120 mg/ml ethanol-d_6.

The I_{2935}/I_{2880} intensity ratio for pure DPPC hydrated with a solution containing 120 mg/ml ethanol-d_6 is lower at 25 and 32 °C and higher at 44 and 48 °C when compared to a similar sample hydrated in a solution containing 20 mg/ml ethanol-d_6. Due to the fact that the I_{2935}/I_{2880} intensity ratio provides information on both the inter- and intrachain characteristics of the acyl chains, the results suggest that there is a decrease in the number of gauche conformers in the acyl chains and a decrease in the level of acyl chain disorder at temperatures of 25 and 32 °C. An increase in the number of gauche conformers in the acyl chains and an increase in the level of acyl chain disorder are observed at temperatures of 44 and 48 °C.

The presence of UDC in the DPPC sample hydrated with a 50 mM KH_2PO_4 solution (initial pH = 5) containing 20 mg/ml ethanol-d_6 results in an increase in both the I_{2850}/I_{2880} and the I_{2935}/I_{2880} intensity ratios over the entire temperature range studied. These data are presented in Figures 3.21 and 3.22. Increases in the I_{2850}/I_{2880} intensity ratio indicate that UDC disorders the lipid's acyl chains, while the increase in the I_{2935}/I_{2880} intensity ratio indicates an increases the number of gauche conformers in the lipid bilayer

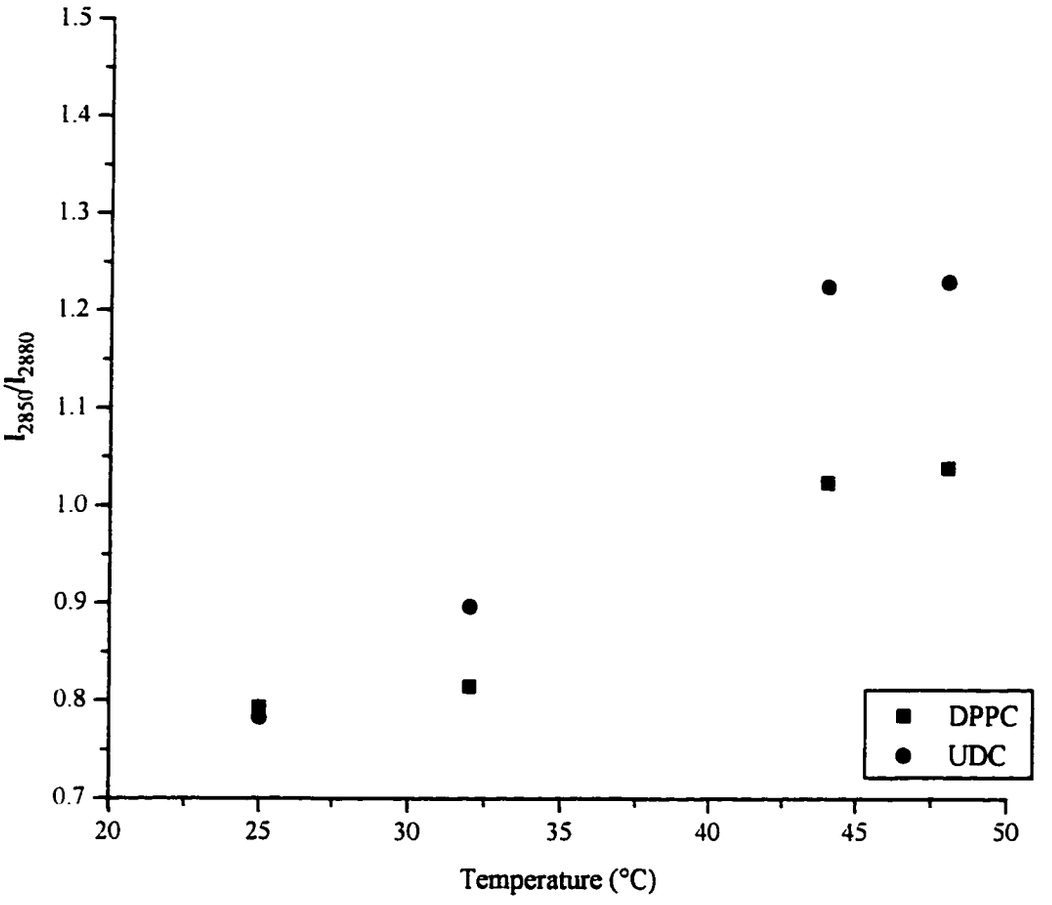


Figure 3.21: Plot of the Ratio of the Intensities at 2850 and 2880 cm^{-1} ((I_{2850}/I_{2880}) versus Temperature for DPPC/UDC Samples Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

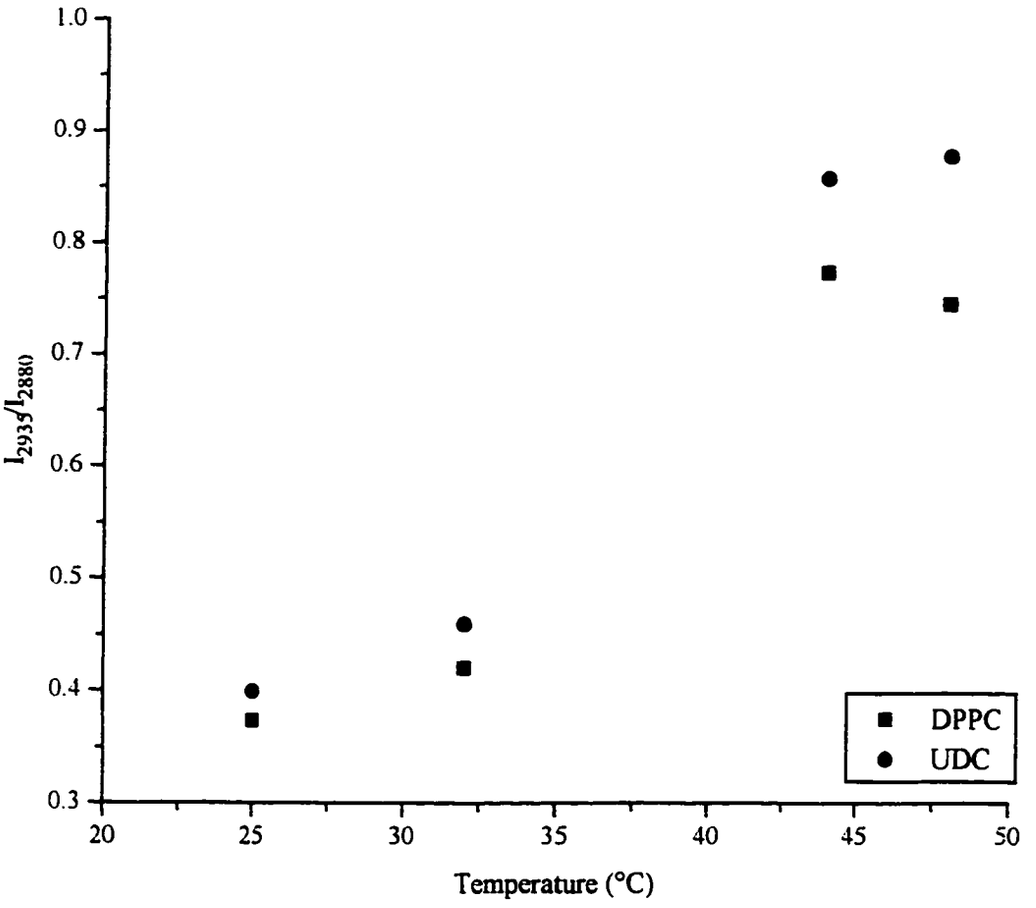


Figure 3.22: Plot of the Ratio of the Intensities at 2935 and 2880 cm^{-1} (I_{2935}/I_{2880}) versus Temperature for DPPC/UDC Samples Hydrated with a 20 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

and an increase in the level of acyl chain disorder in both the gel and the liquid crystalline phases.

Experiments were also performed to determine the effect which increasing the ethanol concentration in the hydrating solution, to 120 mg/ml ethanol-d_6, has on the interaction between UDC and the DPPC bilayer. Data for samples of pure DPPC and a DPPC sample containing 15 mole percent UDC hydrated with a solution containing 120 mg/ml ethanol-d_6 are given in Figures 3.23 and 3.24. The I_{2850}/I_{2880} and I_{2935}/I_{2880} intensity ratios for the UDC containing sample increase significantly for the liquid crystalline phase when compared to the intensity ratios for the pure DPPC sample hydrated under similar conditions. The increased I_{2850}/I_{2880} intensity ratio indicates that the liquid crystalline phase for the UDC containing sample is more disordered than the liquid crystalline phase of pure DPPC hydrated with a similar solution. Only minor changes were observed in the level of acyl chain disorder in the gel phase. The increase in the I_{2935}/I_{2880} intensity ratios for the liquid crystalline phase suggest an increase in the number of gauche conformers and an increase in the level of acyl chain disorder for the UDC containing sample when compared to pure DPPC hydrated under similar conditions.

The results of the Raman experiments differ slightly from those obtained from the infrared spectroscopic experiments. The presence of UDC in the DPPC bilayer hydrated with 50 mM KH_2PO_4 solutions (initial pH = 5) containing 20 mg/ml ethanol-d_6 results in an increase in the level of acyl chain disorder in the liquid crystalline phase but little change in the level of acyl chain order in the gel phase according to the infrared data. The Raman data (I_{2850}/I_{2880} and I_{2935}/I_{2880}) suggest a slight increase in the level of acyl chain

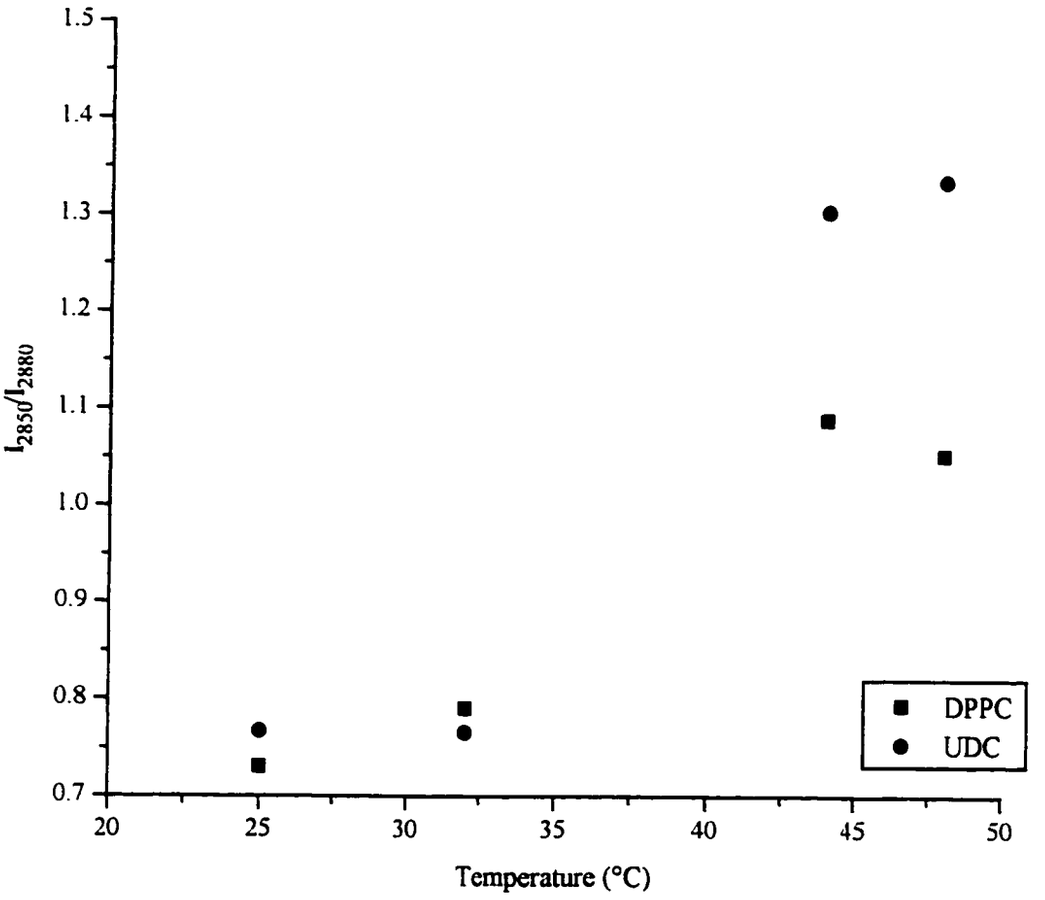


Figure 3.23: Plot of the Ratio of the Intensities at 2850 and 2880 cm^{-1} (I_{2850}/I_{2935}) versus Temperature for DPPC/UDC Sample Hydrated with a 120 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

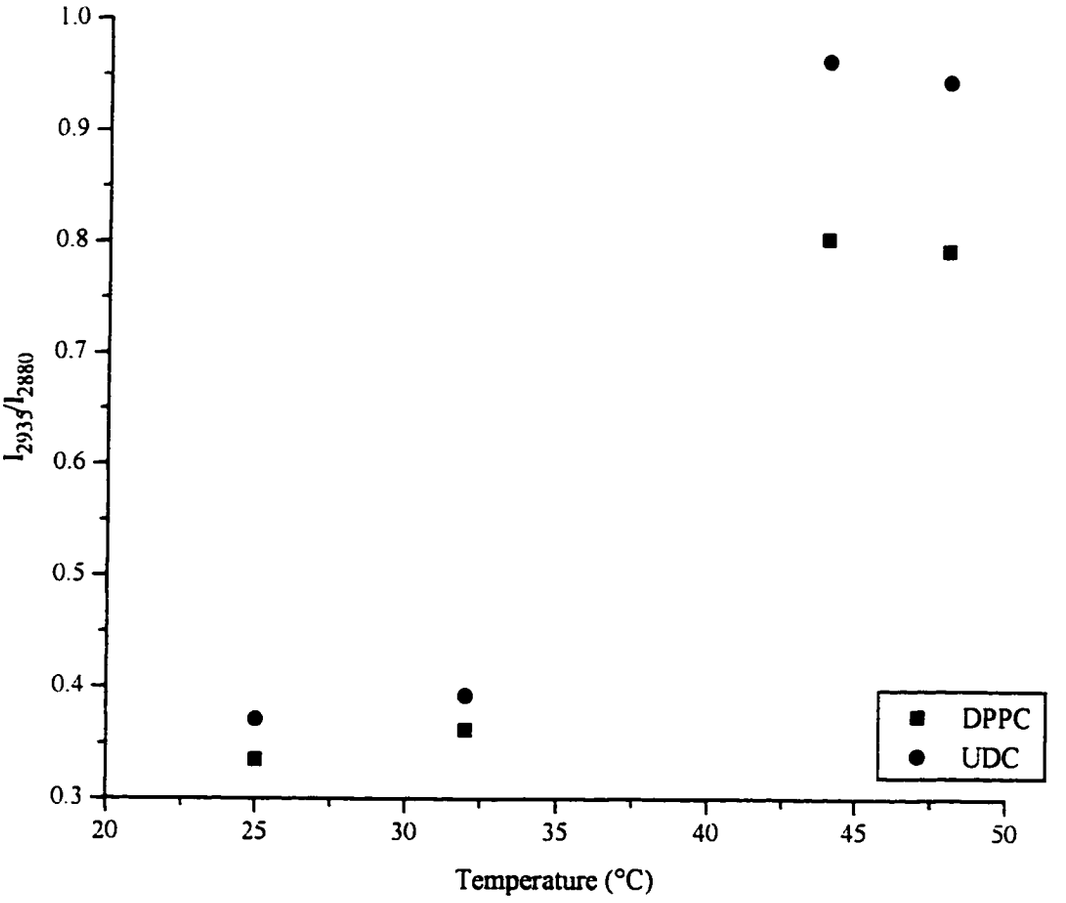


Figure 3.24: Plot of the Ratio of the Intensities at 2935 and 2880 cm^{-1} (I_{2935}/I_{2880}) versus Temperature for DPPC/UDCSamples Hydrated with a 120 mg/ml Ethanol-d_6/50 mM KH_2PO_4 Solution (Initial pH = 5)

disorder for the gel phase and a more significant increase in the level of acyl chain disorder in the liquid crystalline phase. The concentration of ethanol-d_6 in the hydrating solution has a slight affect on the level of acyl chain disorder for pure DPPC in the gel or the liquid crystalline phases based on the Raman data. The infrared data, however, suggest a significant increase in the level of acyl chain disorder in the liquid crystalline phase for pure DPPC hydrated with a 50 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 compared to pure DPPC hydrated with a solution containing 20 mg/ml ethanol-d_6. The data acquired using the two spectroscopic techniques (infrared and Raman) for the UDC containing samples hydrated with a solution having an ethanol-d_6 concentration of 120 mg/ml are similar. That is, using both techniques, the data show a significant increase in the level of acyl chain disorder in the liquid crystalline phase and minor changes in the level of acyl chain disorder in the gel phase for the UDC containing sample when compared to pure DPPC hydrated under similar conditions.

3.4 Conclusion

The results obtained suggest that the effect which the bile salts have on the cooperativity of the phase transitions and the phase transition temperatures are relatively independent of KH_2PO_4 concentration but dependent on the hydrogen ion and ethanol concentration. In general, the presence of bile salt in the lipid assembly reduces the gel to liquid crystalline phase transition temperature indicating a destabilization of the bilayer, reduces the order in the liquid crystalline phase resulting in an increase in the fluidity of this phase and increases the breadth of the phase transition, which indicates a reduction in

the cooperativity between the lipid molecules during the phase transition.

Among the most interesting results are those obtained for samples hydrated with solutions containing ethanol-d_6. Hydrating pure DPPC samples with KH_2PO_4 solutions containing low concentrations of ethanol-d_6 (20 or 55 mg/ml) results in a slight decrease in the phase transition temperature. The phase transition temperature for the DPPC sample hydrated with a solution containing 120 mg/ml ethanol-d_6 (ie., an interdigitated bilayer) increases. The decrease in the phase transition temperature observed in the pure DPPC sample hydrated with a solution containing 20 or 55 mg/ml ethanol-d_6, followed by an increase in the phase transition temperature with increasing ethanol concentration is consistent with the results of studies conducted by many researchers.[95-98, 100] Rowe suggested the decrease in temperature was due to two different interactions; one occurring at low ethanol concentrations and the other, at higher ethanol concentrations.[96] The interaction which occurs in the presence of low concentrations of ethanol is mainly with the fluid, liquid crystalline phase. When higher concentrations of ethanol are present in the lipid bilayer, the interaction occurs predominately with the gel phase. Rowe suggests that at higher concentrations of ethanol, the interaction occurs with the headgroup and stabilizes the membrane thus, causing the transition temperature to shift towards higher values.[96]

The most dramatic effect observed for the UDC and CDC containing samples hydrated with solutions containing ethanol-d_6 is the increase in the cooperativity between the lipid molecules during the phase transition. Even small amounts of ethanol added to the hydrating solution (20 mg/ml) cause a noticeable reduction in the breadth of the phase

transition profile for the bile salt containing samples. Still another striking observation is that the presence of ethanol in the hydrating solution, at concentrations in excess of the level known to produce an interdigitated bilayer structure, at all of the pH's studied, minimizes the effects which the bile salts have on the DPPC bilayer. That is, the phase transition temperatures, the cooperativities of the lipid molecules during the phase transition, and the levels of acyl chain disorder in the liquid crystalline phase for these samples are similar to those obtained for pure DPPC hydrated under the same conditions. It can be concluded, therefore, that ethanol, at high concentrations, minimizes the effects of the bile salts on the lipid bilayer structure.

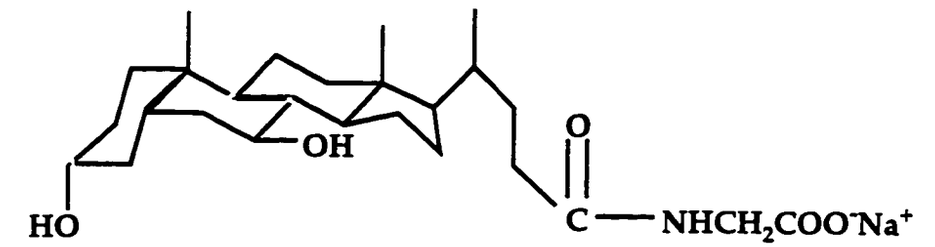
The data obtained for the samples hydrated with ethanol containing solutions may also provide information as to the position at which the bile salt interacts with the lipid assembly. Rowe [96] and Simon et al. [97] concluded that at high ethanol concentrations the ethanol is interacting with the headgroup. If this is occurring, the bile salt may not have the space necessary to interact with the lipid assembly and a competition would develop between the ethanol and the bile salts for the "active" sites. These active sites would be the locations where the two species would interact with the bilayer and cause changes in the stability of the bilayer, the fluidity of the bilayer, and the cooperativity between the lipid molecules during the phase transition. Since the concentration of ethanol molecules in the hydrating solution far exceeds the concentration of bile salt molecules, the ethanol is able to occupy more of these sites. Thus, the effect which the bile salts have on the lipid bilayer is reduced significantly.

CHAPTER 4

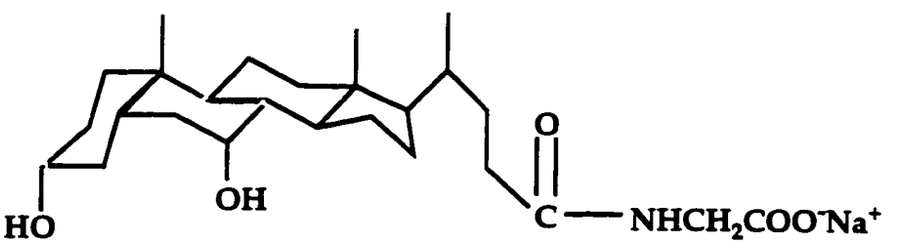
INTERACTIONS OF CONJUGATED BILE SALTS WITH MULTILAMELLAR VESICLES OF DPPC

4.1 Introduction

The results of the previous chapter show that the unconjugated bile salts interact with a DPPC membrane system causing an increase in the fluidity of the liquid crystalline phase, a decrease in the cooperativity between the lipid molecules during the phase transition and a destabilization of the lipid bilayer. The human body contains both the taurine and glycine conjugated bile salts, as well as the unconjugated bile salts, at much lower concentrations. Taurochenodeoxycholate (TCDC) and glycochenodeoxycholate (GCDC) are found, in high concentrations, in normal human bile, while the conjugated UDC bile salts are typically found in patients who are undergoing bile acid therapy with unconjugated UDC.[101] The molecular structures of the glycine and taurine conjugated bile salts are given in Figure 4.1. The bile salts are conjugated with glycine or taurine in the liver and are secreted in bile. The bile salts facilitate digestion and the transport of dietary lipids in the small intestine. Due to a process known as enterohepatic circulation, only a very small quantity of the bile acid pool is lost from the body, . Enterohepatic circulation involves the absorption of bile salts from the terminal ileum, returning the bile salts to the liver, where they are reconjugated and resecreted, via the bile ducts, to the



(a)



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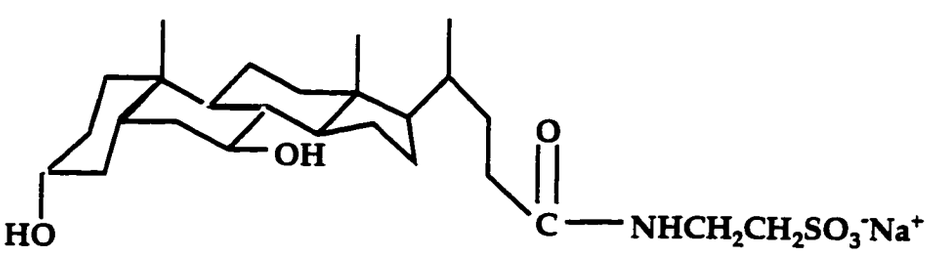

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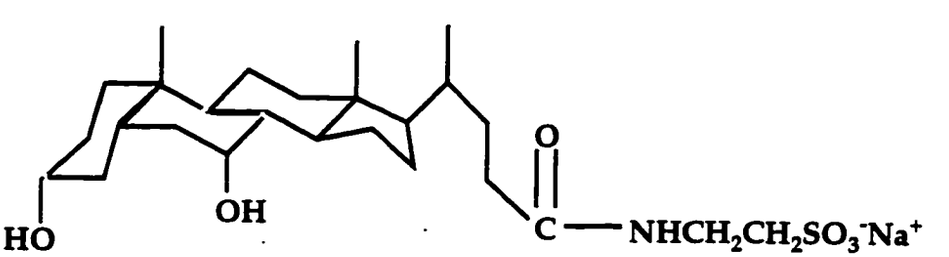

(d)

Figure 4.1: Molecular Structures of a) Sodium Glycoursodeoxycholate, b) Sodium Glycochenodeoxycholate, c) Sodium Tauroursodeoxycholate, and d) Sodium Taurochenodeoxycholate

gallbladder.

Conjugation of the acid functionality with glycine or taurine increases the hydrophilicity of the bile salts.[102] It has been shown that the less toxic bile salts are more hydrophilic.[41] Thus, the conjugated bile salts are known to be less toxic than the corresponding unconjugated bile salts.[41] The taurine and glycine conjugates of the CDC epimer have been shown, however, to affect the flow of bile. [103, 101] The taurine conjugate of UDC has been shown to prevent damage induced by other, more toxic, bile salts, such as taurochenodeoxycholate.[104-106] Glycine conjugated UDC also shows protective properties, however it is not as protective as the taurine conjugate of UDC.[41, 106]

Heuman et al. studied the protective effect of UDC on human erythrocytes.[106] The hemolysis of cells was monitored by measuring changes in the absorbance, due to the release of hemoglobin from the erythrocytes, at 540 nm. The results of these studies showed that more hemolysis occurred when only TCDC was present than when TCDC and tauroursodeoxycholate (TUDC) were present.[106] The higher the mole ratio of TUDC to TCDC, the less hemolysis occurred. The results of these studies also indicated that both TUDC and glycoursodeoxycholate (GUDC) offer "protection" to the membrane, but GUDC provides less protection than TUDC.[106] Thus, it appears that the taurine and the glycine conjugated bile salts have different effects on the membrane.

Guldutuna et al. also investigated the protective effects of UDC and its conjugates on hepatocyte membranes.[44] They used electron paramagnetic spectroscopy to monitor the membrane structure. Results of their experiments showed that when a cell membrane

is incubated with CDC and either TUDC or GUDC, the polarity, in the region surrounding the probe molecule, decreases, thereby, decreasing the damaging effects of CDC on the cell membrane. The polarity of the membrane decreased more with TUDC than with GUDC. The polarity of both the membrane and the bile salts were used to determine the location of the disturbance in the membrane caused by the bile salt. It was found that UDC did not cause changes in the polarity at the membrane interface. TUDC and GUDC did not cause changes in the polarity at the core of the bilayer. Thus, it was postulated that UDC is incorporated into the core of the bilayer and TUDC and GUDC are located with their polar side chains interacting with the membrane surface. Differences in the possible locations of the bile salts in the membrane were suggested as reasons for the differences in the effects which each of the bile salts have on the lipid assembly.

Ethanol is known to partition into the lipid bilayer. The partitioning of ethanol into the bilayer has been shown to cause changes in the physical properties of the lipid assemblies and alteration of the corresponding membrane function.[95] The results of experiments presented in Chapter 3 show that ethanol subdues the effects which the unconjugated bile salts have on multilamellar vesicles of DPPC. Ethanol, when present in samples containing the unconjugated bile salts, increased the stability of the bilayer, increased the cooperativity between the lipid molecules during the phase transition, and increased the fluidity in the liquid crystalline phase.

In the body, bile is secreted into the small intestine when the gall bladder contracts during digestion. The majority of ethanol that enters the body is absorbed in the small intestines.[98] Since bile salts and ethanol can both be present in the small intestine,

studies which monitor the effect which bile salts have on the ethanol/model membrane system may provide important clues to the understanding of the interactions which occur among the bile salts, ethanol, and cellular membranes in the body. By studying the interactions at ethanol concentrations which are both above and below the concentration known to induce interdigitation, the effects which bile salts have on an interdigitated and noninterdigitated lipid bilayer can be determined. The concentration of ethanol that is needed to induce interdigitation is high, but it has been calculated that cells in the body may be exposed to these concentrations during periods of heavy drinking.[98] Therefore, studies performed on interdigitated bilayers may be important for understanding the effects which ethanol has on membranes in the human body.

Due to the high concentration of the glycine conjugated bile salts of UDC and CDC in the human body under some conditions, these bile salts were chosen for this study. The interaction of these conjugated bile salts with a multilamellar model membrane system composed of the phospholipid, DPPC, was studied using hydrating solutions having initial pH's equal to 5, 7, and 8. Fourier transform infrared spectroscopy, a noninvasive technique, was used to monitor structural changes which occur within the lipid bilayer due to the presence of the glycine conjugated forms of UDC and CDC. The frequency of the methylene stretching bands was used to provide information regarding the stability of the membrane as a function of temperature, the cooperativity between the lipid molecules during the gel to liquid crystalline phase transition and the level of intrachain order/disorder (or the fluidity) of the lipid bilayer.

Experiments were also performed to determine the effect which the presence of

ethanol, in the hydrating solutions, has on the interactions between the glycine conjugated bile salts and DPPC. Hydrating solutions having initial pH's of 5, 7, and 8, which contained 0, 20 and 120 mg/ml of ethanol-d_6 were utilized.

4.2 Materials and Methods

4.2.1 Materials

Synthetic 1,2 dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (manufacturer stated purity > 99 %) was purchased, in powder form, from Avanti Polar Lipids, Inc. (Alabaster, AL). DPPC from lot number 160PC-201 was used for these studies. Sodium glycoursodeoxycholate (GUDC) (manufacturer stated purity 97 %, by TLC) and sodium glycochenodeoxycholate (GCDC) (manufacturer stated purity 95 %, by TLC) were obtained from Calbiochem (La Jolla, CA). All of the bile salts were used as received without further purification. Fully deuterated ethanol (ethanol-d_6, manufacturer stated purity 99+ D %) was purchased from Sigma Chemical Company (St. Louis, MO). Potassium phosphate monobasic was purchased from Fisher Scientific (Pittsburgh, PA). Potassium hydroxide (KOH), in pellet form, was obtained from J.T. Baker Chemical Company (Pittsburgh, PA). Laboratory distilled, deionized water was used for the preparation of all solutions.

4.2.2. Sample Preparation

The procedures used to prepare the lipid samples hydrated with KH_2PO_4 solutions having initial pH's of 5, 7, and 8 and ethanol-d_6 concentrations of 0, 20 and 120 mg/ml as

described in Chapter 3.

4.2.3 Data Acquisition

The spectral data were acquired and analyzed as described in Chapter 2. Previous results based on the reproducibility of these lipid experiments have shown that changes in the frequency of the methylene symmetric stretching band which are less than 0.1 cm^{-1} are not considered to be significant. The results of experiments discussed in Chapter 2 determined that the spectral contribution of the unconjugated bile salts to the frequency of the methylene symmetric stretching band of the lipid, at the bile salt concentrations studied, is small (ie., 0.01 cm^{-1} in the gel phase and by 0.03 cm^{-1} in the liquid crystalline phase). Results presented in Chapter 3 show that the addition of 120 mg/ml ethanol-d_6 to the hydrating solutions shifts the frequency of the methylene symmetric stretching bands by +0.06 cm^{-1} in the gel phase and by +0.05 cm^{-1} in the liquid crystalline phase. These values are within our level of reproducibility. The spectral contribution of the glycine conjugated bile salts to the frequency of the methylene symmetric stretching band should also be small since the spectra of the glycine conjugated and unconjugated bile salts are similar in this region of the spectrum.

4.3 Results and Discussion

The frequency of the methylene stretching bands of the lipid's acyl chains was used to provide information on the ratio of gauche/trans conformers or acyl chain order, in the hydrocarbon chains of the lipids.[4, 13, 14] As the number of gauche conformers in the

lipid's hydrocarbon chains increase, the frequencies of the symmetric and asymmetric methylene stretching bands located at approximately 2850 cm^{-1} and 2920 cm^{-1}, respectively, also increase. An increase in the number of gauche conformers results in a more fluid, more "disordered" phase, while conversely a decrease in the number of gauche conformers results in a less fluid, more "ordered" phase.

The effects which the glycine conjugated forms of UDC and CDC have on a model membrane composed of the phospholipid DPPC were investigated. Data were acquired for samples immediately after preparation ("fresh") and again after five days. The samples were held at room temperature during the five days required for the experiments. Changes in the level of acyl chain disorder were observed in the phase transition profiles obtained for some of the samples containing either of the glycine conjugated bile salts over time. In general, the level of acyl chain disorder decreased with time. The instability of the bilayer with time was not observed in previous studies which utilized the unconjugated forms of the bile salts. Samples containing the glycine conjugated bile salts appeared to be more stable after 5 days than the fresh samples. Therefore, the data presented for all of the samples (pure DPPC and DPPC containing the glycine conjugated bile salts) are for 5 day old samples.

4.3.1 Effect of Glycine Conjugated Bile Salts on Multilamellar DPPC Assemblies Hydrated with Solutions Having Initial pH's of 5, 7, and 8

Experiments were performed to determine the effects which the glycine conjugates of UDC and CDC have on the DPPC bilayer. The phase transition profiles for pure

DPPC, DPPC with 15 mole percent GUDC and DPPC with 15 mole percent GCDC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 are given in Figure 4.2. The frequency of the methylene symmetric stretching band shifts to higher wavenumber in the liquid crystalline phase for both the GUDC and the GCDC containing sample. The frequency of the methylene symmetric stretching mode increases by 0.2 cm^{-1} for the GUDC containing sample and by 0.4 cm^{-1} for the GCDC containing sample relative to the frequency of the methylene symmetric stretching band of pure DPPC in the liquid crystalline phase. The shift in the frequency indicates that the level of disorder of the lipid's acyl chains and the fluidity of the liquid crystalline phase increase with the addition of either bile salt. No significant change in the level of acyl chain order in the gel phase is observed for either the GUDC or the GCDC containing samples. The phase transition profile for the GCDC containing sample shows biphasic characteristics. Similar biphasic profiles were observed for samples hydrated with a KH_2PO_4 solution having an initial pH of 8 and containing the unconjugated form of UDC and CDC. The two phase transitions occur at approximately 35.0 °C and 39.8 °C. The breadths of the two phase transitions are very different. The transition which occurs at approximately 35.0 °C is sharp and has a breadth of 0.8 °C, while the breadth for the transition at approximately 39.8 °C is 5.3 °C. The breadths of the two phase transitions indicate that the cooperativity between the lipid molecules is greater for the lower temperature phase transition than the higher temperature phase transition. When GUDC is present in the DPPC bilayer, the breadth of the gel to liquid crystalline phase transition increases. This increase in the breadth of the phase transition indicates that the presence of GUDC decreases the cooperativity between

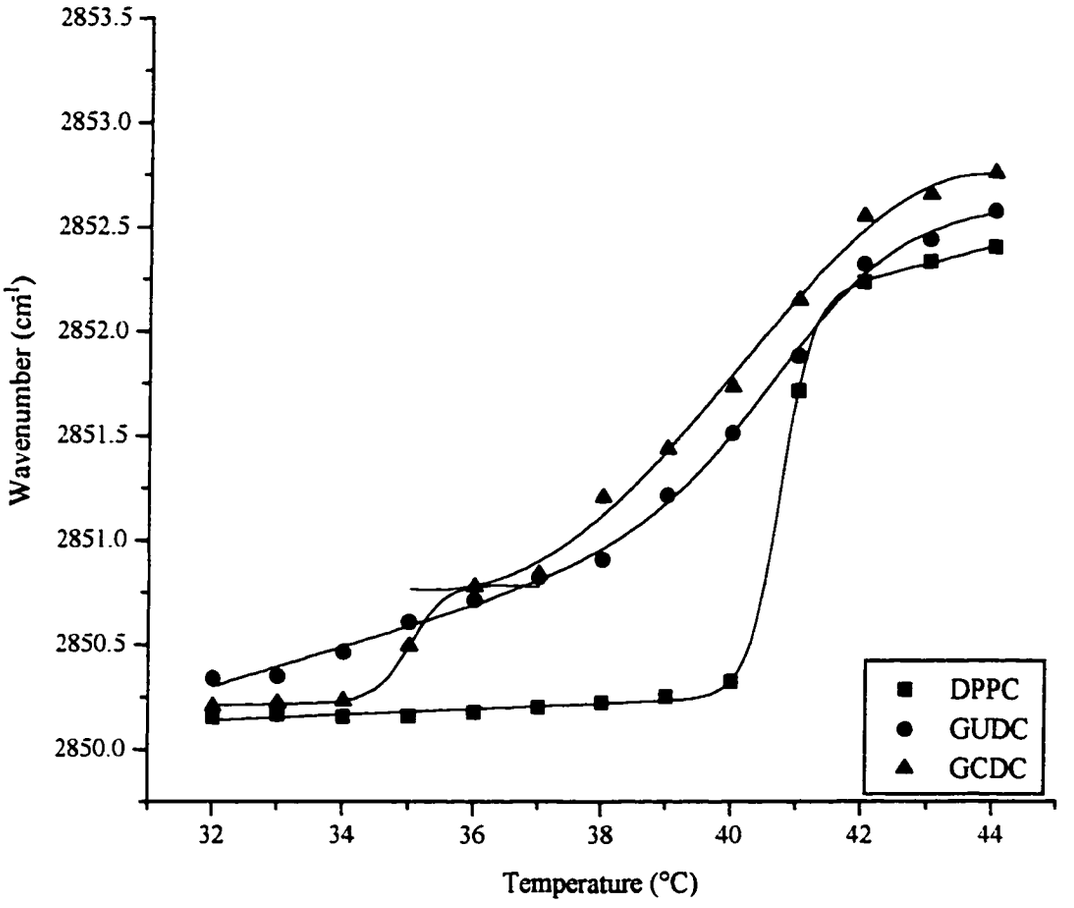

Figure 4.2 Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7)

the lipid molecules of the bilayer during the phase transition. The gel to liquid crystalline phase transition temperature for the GUDC containing sample also decreases relative to the phase transition temperature of pure DPPC indicating the presence of GUDC destabilizes the lipid bilayer. The phase transition parameters obtained from these experiments are given in Table 4.1. Spectra were acquired for a GUDC containing sample hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 using both an increasing temperature and a decreasing temperature ramp. The phase transition profiles obtained for DPPC containing 15 mole percent GUDC hydrated with a 200 mM KH_2PO_4 solution (initial pH = 7) are given in Figure 4.3. No changes were observed in the phase transition profiles obtained from the two sets of data.

Experiments were performed to determine the effect which lowering the pH of the hydrating solution has on the interactions between the glycine conjugated bile salts and the DPPC lipid assembly. Phase transition profiles for DPPC samples, with and without the presence of either GUDC or GCDC, hydrated with a solution having an initial pH of 5 are given in Figure 4.4. The level of acyl chain disorder in the gel phase for the samples containing either GUDC or GCDC increases relative to that of pure DPPC as indicated by the increase in the frequency of the methylene symmetric stretching band for these samples. The frequency of the methylene symmetric stretching band increases, by 0.2 cm^{-1}, for both of the bile salt containing samples. The frequency of the methylene symmetric stretching mode also increases in the liquid crystalline phase for DPPC samples containing either GUDC or GCDC under these hydrating conditions. The frequency of the methylene symmetric stretching band of the lipid's acyl chains in the liquid crystalline phase increases

Table 4.1: Phase Transition Paramters for Samples Hydrated with 200 mM KH_2PO_4 Solutions Having an Initial pH of 7

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2850.2	2849.9	2850.1
	44 °C*	2852.4	2852.3	2853.1
	ΔT (°C)	0.9	0.7	0.8
	ΔT_m (°C)**		0.4	-0.1
GUDC	32 °C*	2850.3	2850.2	2850.1
	44 °C*	2852.6	2852.6	2853.2
	ΔT (°C)	4.0	1.8	0.6
	ΔT_m (°C)**	0.3	2.9	0.4
GCDC	32 °C*	2850.2	2849.9	2850.0
	44 °C*	2852.8	2852.7	2853.1
	ΔT (C)	0.8[a], 5.3[b]	1.8	1.2
	ΔT_m (°C)**	5.7[a], 0.9[b]	3.0	0.9

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC(0\ mg/ml\ ETOH\text{-}d6)} - T_{Bdo\ Salt}$)

[a] Data acquired for the first phase transition

[b] Data acquired for the second phase transition

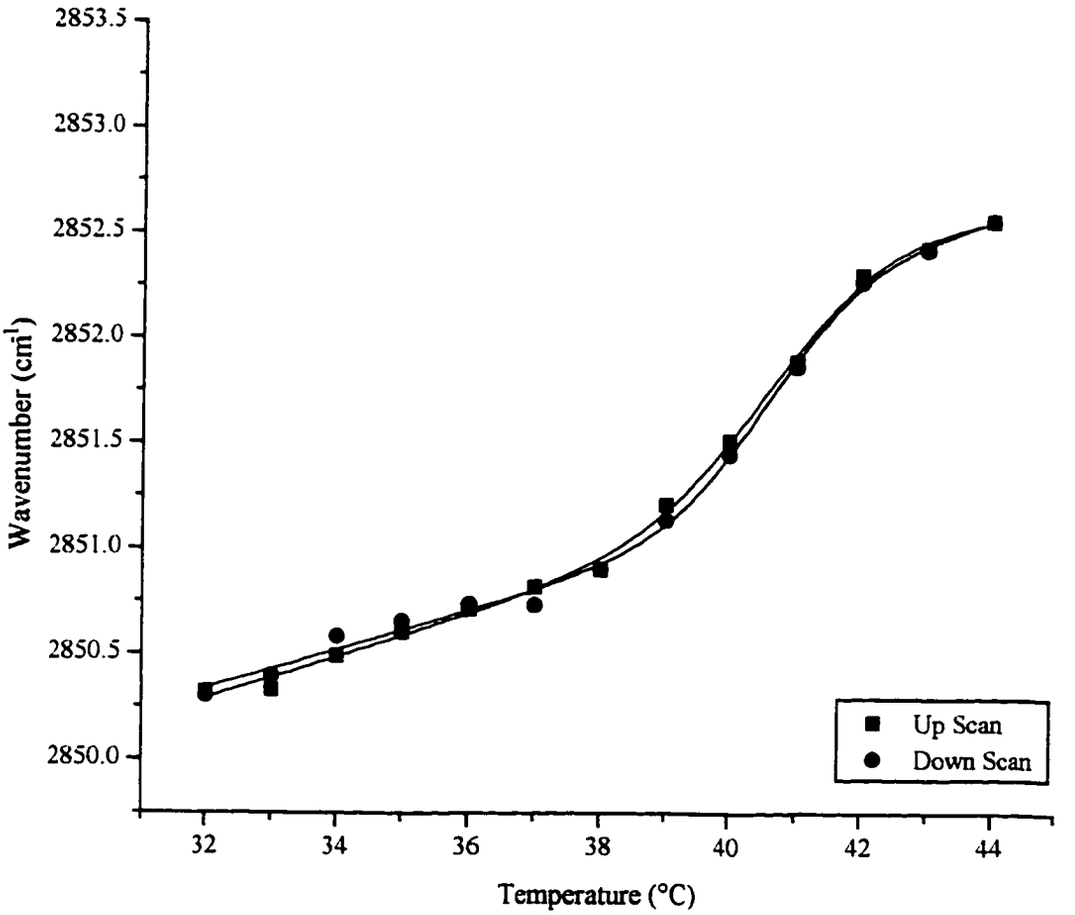

Figure 4.3: Phase Transition Profiles for DPPC/GUDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7)

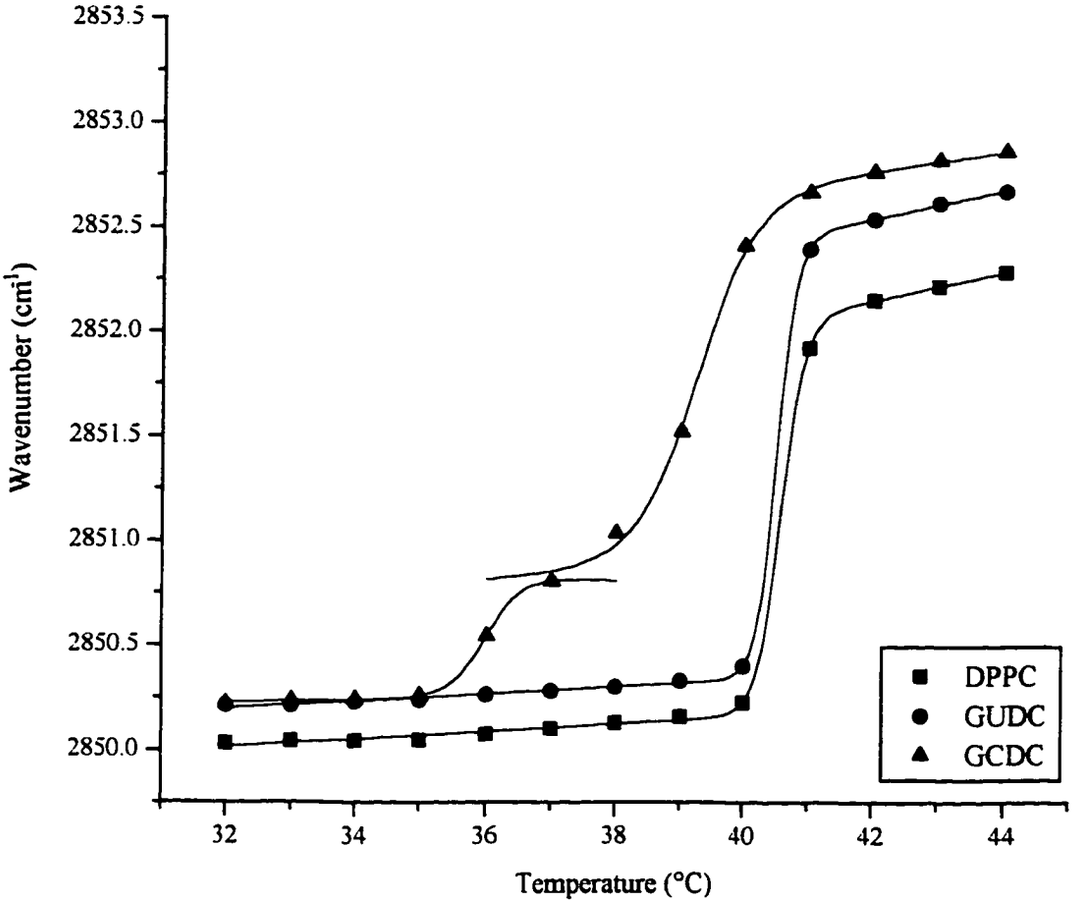

Figure 4.4: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 5)

by 0.4 cm^{-1} and by 0.6 cm^{-1} for the GUDC and GCDC containing samples, respectively. The phase transition profile for the GCDC containing sample shows biphasic characteristics with two apparent phase transitions one occurring at approximately 36.0 °C and a second, at 39.3 °C. The breadths of the two phase transitions were determined to be 1.2 °C and 2.9 °C indicating that the cooperativity between the lipid molecules is less for the transition which occurs at the higher temperatures. The breadth of the GUDC containing sample did not change significantly from the breadth obtained for pure DPPC hydrated under similar conditions. The phase transition temperature for the GUDC containing sample does not change significantly when compared to that of pure DPPC hydrated under similar conditions. The phase transition parameters are summarized in Table 4.2.

The effect which increasing the initial pH of the hydrating solution has on the interactions between the glycine conjugated bile salts and the DPPC bilayer were determined. A 200 mM KH_2PO_4 hydrating solution having an initial pH of 8 was used for this study. The phase transition profiles for pure DPPC and DPPC with 15 mole percent of either GUDC or GCDC are given in Figure 4.5. The level of acyl chain disorder in the gel phase increases for the GUDC containing sample as indicated by an increase of 0.3 cm^{-1} in the frequency of the methylene symmetric stretching band. The level of acyl chain disorder in the gel phase for the GCDC containing sample does not change significantly from that of pure DPPC. The level of acyl chain disorder in the liquid crystalline phase for the samples containing GUDC or GCDC increases as indicated by an increase of 0.2 cm^{-1} in the frequency of the methylene symmetric stretching band. Thus, the fluidity of the

Table 4.2: Phase Transition Paramters for Samples Hydrated with 200 mM KH_2PO_4 Solutions Having an Initial pH of 5

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2850.0	2850.0	2849.8
	44 °C*	2852.3	2852.5	2852.6
	ΔT (°C)	0.9	1.0	0.5
	ΔT_m (°C)**		0.6	0.0
GUDC	32 °C*	2850.2	2850.2	2850.0
	44 °C*	2852.7	2852.8	2853.2
	ΔT (°C)	0.6	0.3	0.4
	ΔT_m (°C)**	0.1	1.3	0.1
GCDC	32 °C*	2850.2	2850.2	2849.9
	44 °C*	2852.9	2853.0	2853.0
	ΔT (°C)	1.2[a], 2.9[b]	1.7	0.4
	ΔT_m (°C)**	4.6[a], 1.3[b]	2.5	1.3

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC(0\ mg/ml\ ETOH\text{-}d6)} - T_{Bile\ Salt}$)

[a] Data acquired for the first phase transition

[b] Data acquired for the second phase transition

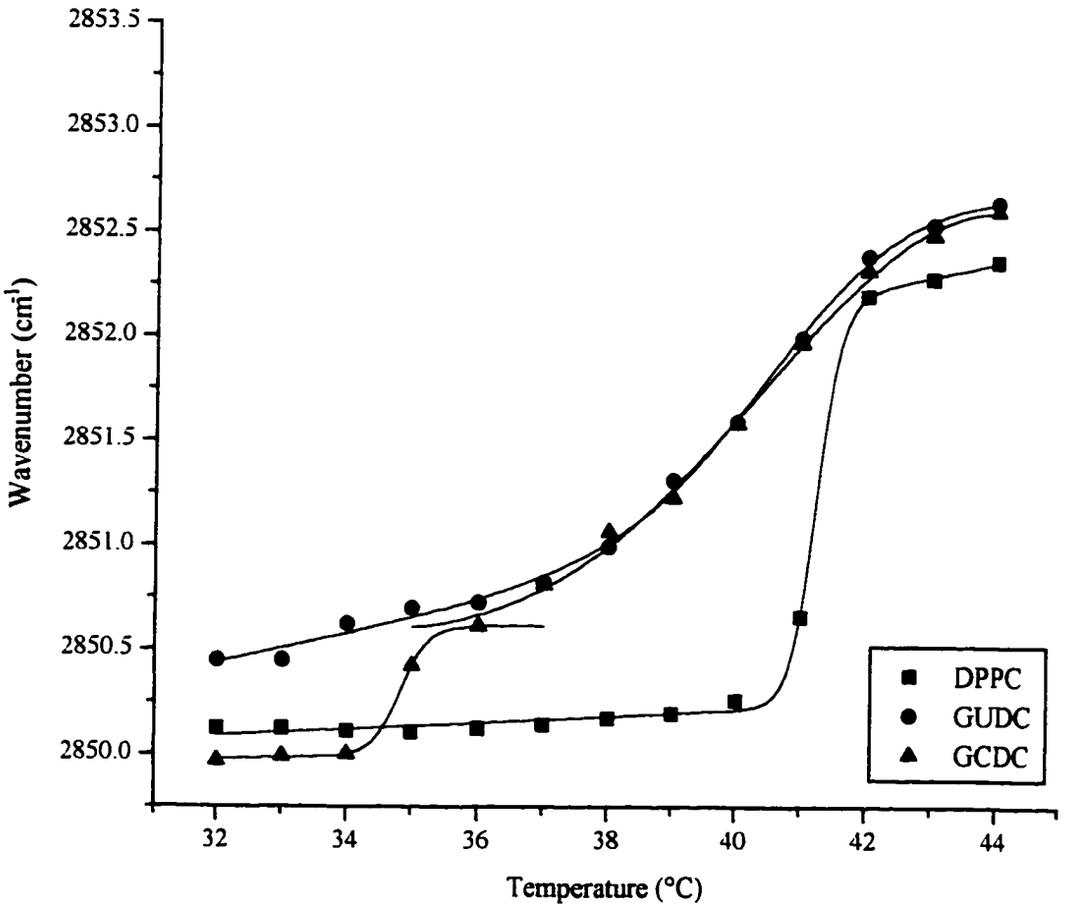

Figure 4.5: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 8)

gel phase increases for the sample containing GUDC, while the fluidity of the liquid crystalline phase increases for both bile salt containing samples. The phase transition profile obtained for the GCDC containing sample shows biphasic characteristics with phase transitions occurring at approximately 34.8 °C and 40.6 °C. The breadths of the two phase transitions are 0.8 °C and 3.6 °C, respectively. The breadths of the phase transition for the DPPC sample which contains GUDC increase relative to that of pure DPPC hydrated under similar conditions. The phase transition temperatures for the GUDC containing sample decrease by approximately 1.1 °C compared to pure DPPC. Thus, the addition of either GCDC or GUDC to pure DPPC destabilizes the bilayer. The phase transition parameters are summarized in Table 4.3.

4.3.2 Effect of Adding Ethanol-d_6 to Hydrating Solutions Having Initial pH's of 5, 7 and 8

The effect which adding ethanol-d_6 to the KH_2PO_4 hydrating solution has on the glycine conjugated bile salt/DPPC interactions was determined. The phase transition profile for pure DPPC hydrated with a solution containing no ethanol-d_6, along with the profiles for DPPC, DPPC with GUDC and DPPC with GCDC hydrated with a solution containing 20 mg/ml ethanol-d_6 (initial pH = 7) are given in Figure 4.6. The frequency of the methylene symmetric stretching band in the gel phase decreases, by 0.3 cm^{-1}, for the pure DPPC sample and the DPPC sample containing 15 mole percent GCDC hydrated with an ethanol-d_6 containing solution compared to similar samples hydrated with a solution which does not contain ethanol-d_6. The decrease in the frequency of the

Table 4.3: Phase Transition Paramters for Samples Hydrated with 200 mM KH_2PO_4 Solutions Having an Initial pH of 8

		0 mg/ml ETOH-d_6	20 mg/ml ETOH-d_6	120 mg/ml ETOH-d_6
DPPC	32 °C*	2850.1	2850.0	2850.0
	44 °C*	2852.4	2852.4	2853.1
	ΔT (°C)	0.8	0.7	0.6
	ΔT_m (°C)**		1.7	-0.1
GUDC	32 °C*	2850.4	2850.0	2849.9
	44 °C*	2852.6	2852.5	2853.0
	ΔT (°C)	4.8	1.4	0.2
	ΔT_m (°C)**	1.1	2.6	1.0
GCDC	32 °C*	2850.0	2850.1	2849.9
	44 °C*	2852.6	2852.7	2852.8
	ΔT (°C)	0.8[a], 3.6[b]	1.6	0.4
	ΔT_m (°C)**	6.4[a], 0.6[b]	3.4	1.8

* Values in wavenumbers (cm^{-1})

** Values equal to ($T_{DPPC(0\ mg/ml\ ETOH\text{-}d6)} - T_{Bile\ Salt}$)

[a] Data acquired for the first phase transition

[b] Data acquired for the second phase transition

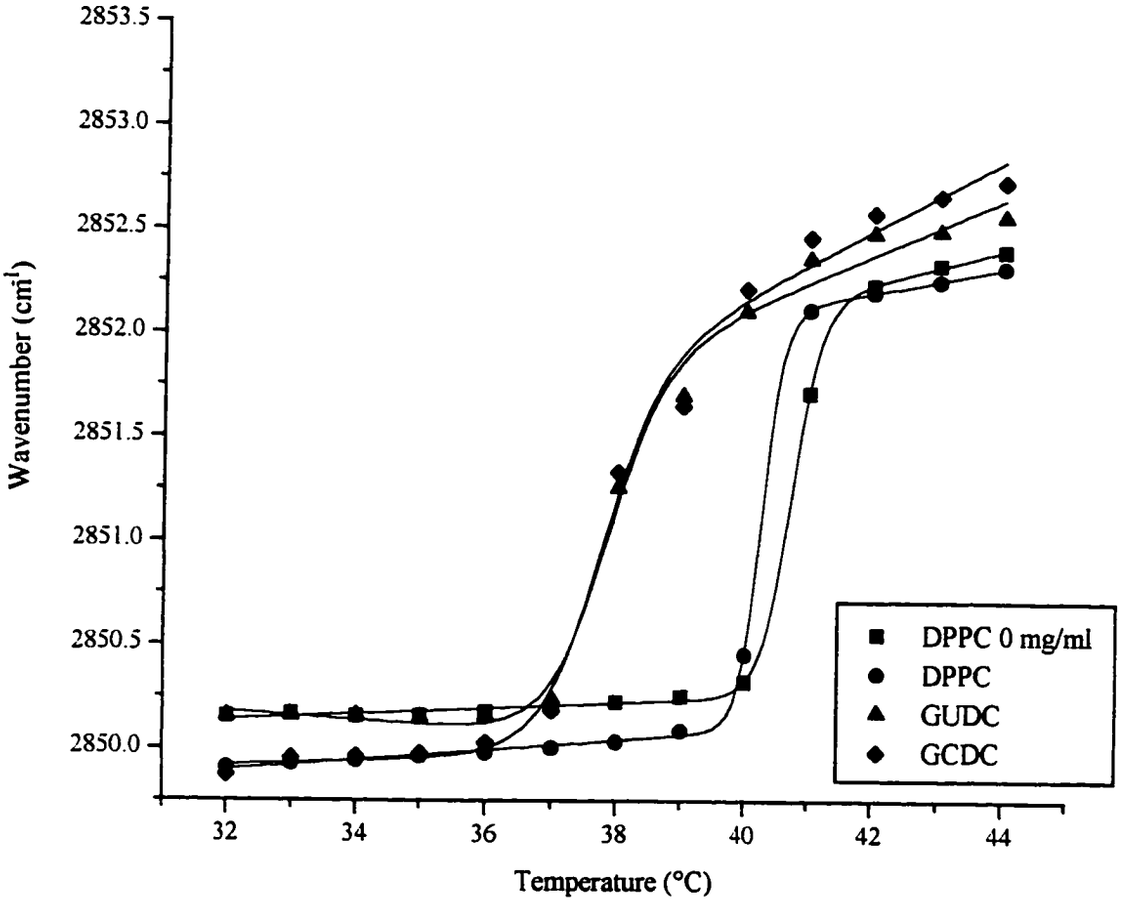

Figure 4.6: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 7)

methylene symmetric stretching band indicates a decrease in the level of acyl chain disorder. The DPPC sample with 15 mole percent GUDC, however, does not show a change in the level of acyl chain disorder when compared to GUDC samples hydrated with a solution containing no ethanol-d_6. The frequency of the methylene symmetric stretching band in the liquid crystalline phase and thus the level of acyl chain disorder does not change significantly for any of the samples hydrated with a solution containing 20 mg/ml ethanol-d_6 compared to similar samples hydrated with a solution containing 0 mg/ml ethanol-d_6. The phase transition temperatures decrease for the bile salt containing samples. The phase transition temperature for the GUDC containing sample hydrated with a solution containing 20 mg/ml ethanol-d_6 decreases by 2.6 °C when compared to GUDC containing samples hydrated with a solution which does not contain ethanol-d_6. The addition of 20 mg/ml ethanol-d_6 to the hydrating solution results in the breadths of the DPPC gel to liquid crystalline phase transitions decreasing for the bile salt containing samples when compared to similar bile salt containing samples hydrated with a solution which does not contain ethanol-d_6. The breadth of the phase transition decreases from 4.0 °C to 1.8 °C for the GUDC containing sample. The breadths of the phase transitions, the frequencies of the methylene symmetric stretching modes and the ΔT_m's are given in Table 4.1.

To determine if increasing the concentration of ethanol-d_6 in the hydrating solution affects the interactions which the glycine conjugated bile salts have with the DPPC bilayer, experiments were performed using KH_2PO_4 hydrating solutions (initial pH = 7) containing 120 mg/ml ethanol-d_6. Phase transition profiles for samples hydrated with a 200 mM

KH_2PO_4 solution having an initial pH of 7 and containing 120 mg/ml ethanol-d_6 are given in Figure 4.7. The frequency of the methylene symmetric stretching band, in the gel phase, increases for the pure DPPC sample, by 0.2 cm^{-1}, compared to the pure DPPC sample hydrated with a solution containing 20 mg/ml ethanol-d_6. Thus, higher concentrations of ethanol-d_6 in the hydrating solutions result in an increase in the level of disorder of the lipid's acyl chains in the gel phase of the DPPC bilayer when no bile salt is added. No significant changes are observed in the frequency of the methylene symmetric stretching band in the gel phase for DPPC samples containing GUDC or GCDC compared to GUDC or GCDC containing samples hydrated with a solution containing 20 mg/ml ethanol-d_6. The level of acyl chain disorder in the liquid crystalline phase increases for all samples when compared to the samples hydrated with solutions containing either 0 or 20 mg/ml ethanol-d_6. The increase in the level of acyl chain disorder is indicated by the shift in the frequency of the methylene symmetric stretching band to higher wavenumbers, 0.8 cm^{-1}, 0.6 cm^{-1} and 0.4 cm^{-1} for the pure DPPC sample, the DPPC sample with 15 mole percent GUDC and the DPPC sample with 15 mole percent GCDC, respectively, compared to similar samples hydrated with a solution containing 20 mg/ml of ethanol-d_6. The increase in the frequency of the methylene symmetric stretching band in the liquid crystalline phase for these samples indicates that this phase, under these hydrating conditions, is more fluid. The phase transition temperatures for the bile salt containing samples increased compared to the values obtained for the samples hydrated with solutions containing either 0 or 20 mg/ml ethanol-d_6. The phase transition temperatures increased by 1.2 °C and 0.6 °C for the GUDC and GCDC containing samples, respectively, when compared to similar

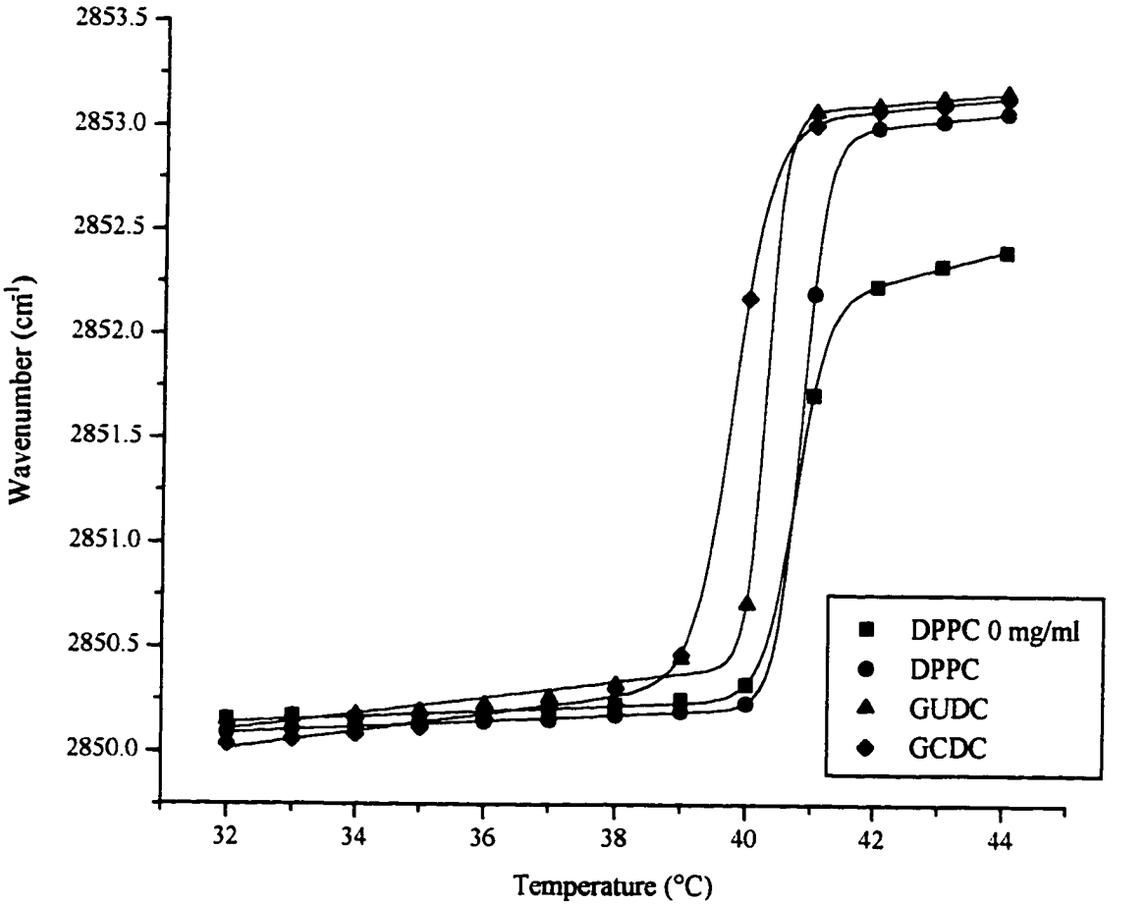

Figure 4.7: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 7)

samples hydrated with a solution containing 20 mg/ml ethanol-d_6. The breadths of the phase transitions for the bile salt containing samples hydrated with this solution decreased when compared to the samples hydrated with solutions containing 0 or 20 mg/ml ethanol-d_6. Thus, the presence of higher concentrations of ethanol-d_6 in the hydrating solution increases the stability of the bilayer and the cooperativity between the lipid molecules during the phase transition for the bile salt containing samples. The phase transition parameters are given in Table 4.1.

The effect which the presence of ethanol-d_6, in the hydrating solution, has on the interactions of the glycine conjugated bile salts hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 5 was determined. The phase transition profiles for DPPC with and without the addition of GUDC or GCDC hydrated with a solution containing 20 mg/ml ethanol-d_6 are given in Figure 4.8. The frequency of the methylene symmetric stretching band for the gel phase did not change for any of the samples when hydrating the lipid with a solution of 200 mM KH_2PO_4 (initial pH of 5) containing 20 mg/ml ethanol-d_6 in comparison to similar samples hydrated with a similar solution which did not contain ethanol-d_6. The frequency of the methylene symmetric stretching band does change for the liquid crystalline phase of pure DPPC. The frequency increases by 0.2 cm^{-1}, indicating an increase in the level of acyl chain disorder, when compared to pure DPPC hydrated with a solution containing no ethanol-d_6. There is no change in the level of acyl chain disorder in the liquid crystalline phase for the bile salt containing samples. The phase transition temperatures decrease for all samples hydrated with a solution containing 20 mg/ml ethanol-d_6 in comparison to similar samples hydrated with a solution which does

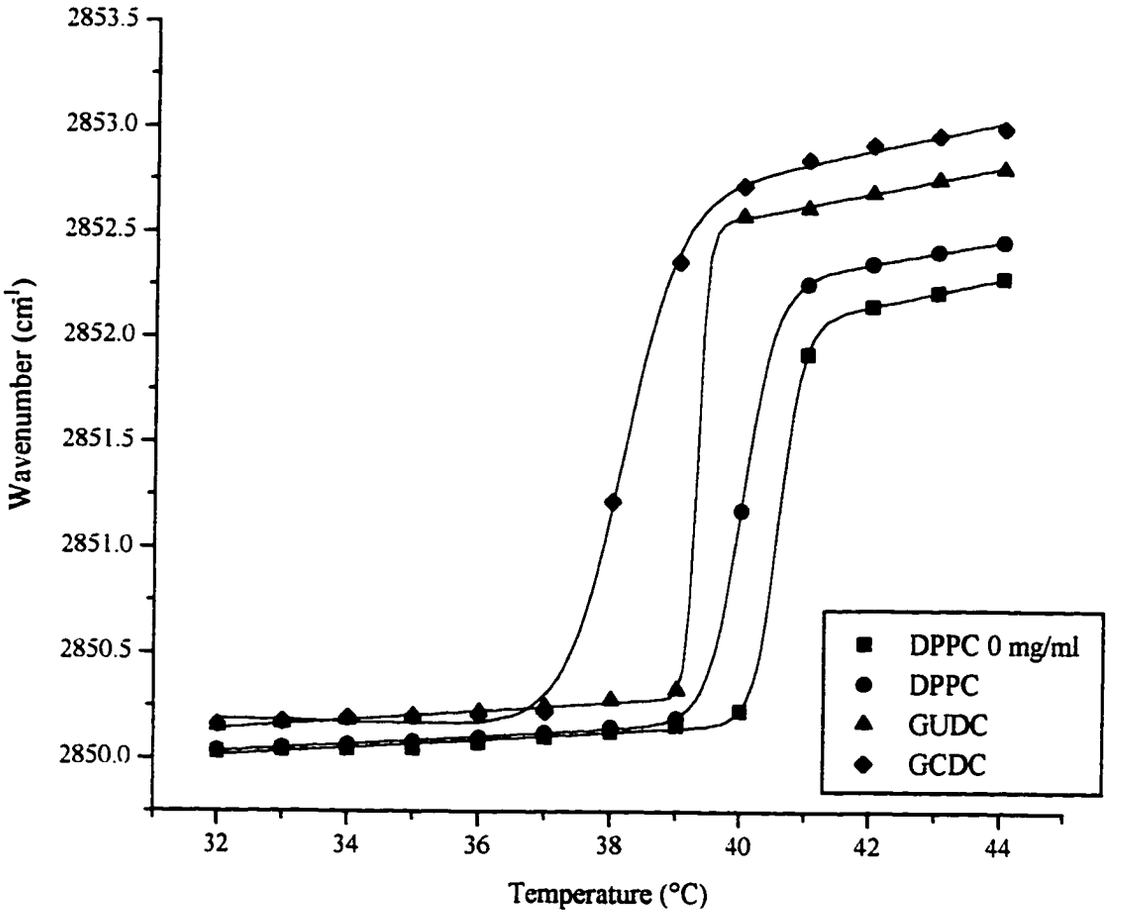

Figure 4.8: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 5)

not contain any ethanol-d_6. The breadths of the phase transitions increase for the bile salt containing samples. The GCDC containing samples loses its biphasic characteristics when ethanol-d_6 is added to the hydrating solution. The phase transition parameters are given in Table 4.2.

Upon adding higher concentrations, 120 mg/ml, of ethanol-d_6 to the hydrating solution, the frequency of the methylene symmetric stretching band decreases in the gel phase for all samples when compared to similar samples hydrated with a solution containing 20 mg/ml ethanol-d_6. These results differ from those obtained for samples containing unconjugated UDC or CDC hydrated with a similar solution. No change in the frequency of the methylene symmetric stretching band was observed for the samples containing the unconjugated bile salts hydrated with a 200 mM KH_2PO_4 (initial pH = 8) solution containing 120 mg/ml ethanol-d_6. The phase transition profiles, for samples hydrated with a solution containing 120 mg/ml ethanol-d_6 are given in Figure 4.9 and the phase transition data are given in Table 4.2. The frequency decreases by 0.2 cm^{-1} for the pure DPPC and the GUDC containing samples hydrated with a solution containing 120 mg/ml ethanol-d_6 and by 0.3 cm^{-1} for the GCDC containing samples. The phase transition temperatures for the bile salt containing samples increase and are within at least 2.4 °C of the phase transition temperature of pure DPPC. The phase transition temperature of the GCDC containing sample is lower than that observed for the GUDC containing sample suggesting that GCDC destabilizes the bilayer to a greater extent than GUDC. The breadth of the phase transition for the GCDC containing sample decreases indicating the cooperativity between the lipid molecules during the phase transition has increased when

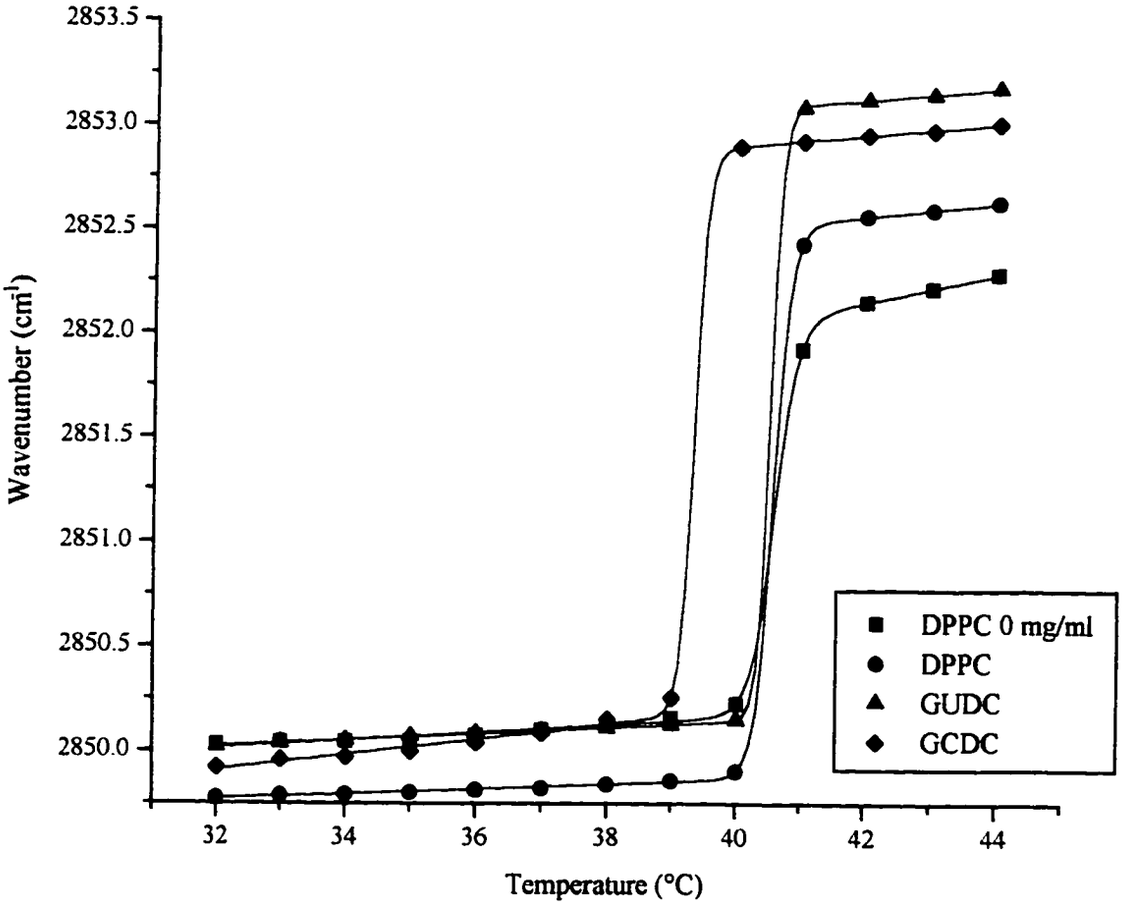

Figure 4.9: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 5)

compared to the GCDC containing samples hydrated with solutions containing either 0 or 20 mg/ml ethanol-d_6.

The effect which the addition of ethanol-d_6 has on the interactions of glycine conjugated bile salts with DPPC bilayers hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 8 was determined. Phase transition profiles for pure DPPC, DPPC with GUDC and DPPC with GCDC hydrated with a solution containing 20 mg/ml ethanol-d_6 are given in Figure 4.10. The level of acyl chain disorder in the gel phase decreases for the GUDC containing sample when compared to the sample hydrated with a solution which does not contain ethanol-d_6, as indicated by the decrease in frequency of the methylene symmetric stretching band. The frequency decreases by 0.4 cm^{-1} for the sample hydrated with a solution containing 20 mg/ml ethanol-d_6 compared to a similar sample hydrated with a solution containing no ethanol-d_6. The decrease in the frequency of the methylene symmetric stretching band for this sample is large, therefore a new sample of DPPC containing 15 mole percent GUDC hydrated with a 200 mM KH_2PO_4 (initial pH = 8) solution containing 120 mg/ml ethanol-d_6 was prepared and analyzed. Similar results were obtained for the second sample. There is no change in the level of acyl chain disorder in the liquid crystalline phase for any of the samples hydrated with a solution containing 20 mg/ml ethanol-d_6 in comparison to similar samples hydrated with a solution which does not contain ethanol-d_6. The phase transition temperatures have decreased for the all samples hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 when compared to similar samples hydrated with solution which does not contain any ethanol-d_6. The phase transition temperatures are 1.7 °C, 1.5 °C and 2.8 °C lower for the

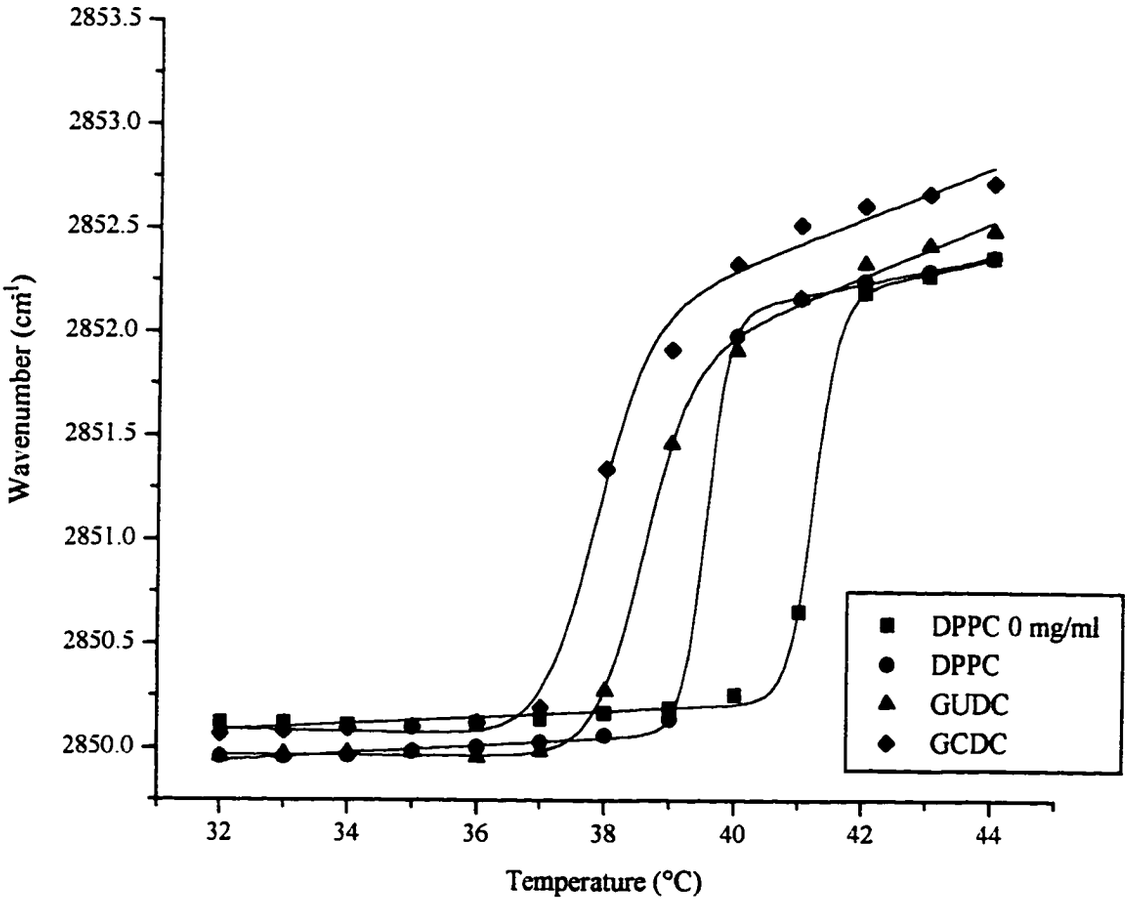

Figure 4.10: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 20 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 8)

pure DPPC, DPPC with 15 mole percent GUDC and DPPC with 15 mole percent GCDC samples, respectively, hydrated with a solution containing 20 mg/ml ethanol-d_6 when compared to similar samples hydrated with a similar solution containing no ethanol-d_6. The breadths of the phase transitions for the bile salt containing samples increase from the breadths obtained for the bile salt containing samples hydrated with a solution which contains no ethanol-d_6. The ΔT_m's, ΔT's and the frequencies of the methylene symmetric stretching bands for pure DPPC and samples containing the glycine conjugated bile salts hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6 (initial pH = 8) are given in Table 4.3.

Experiments were performed to ascertain the effect which increasing the ethanol-d_6 concentration, in the hydrating solution, has on the interactions between the glycine conjugated bile salts and DPPC. The addition of 120 mg/ml ethanol-d_6 to the 200 mM KH_2PO_4 hydrating solution having an initial pH of 8 results in an increase in the frequency of the methylene symmetric stretching band for the pure DPPC and the GUDC containing samples in the liquid crystalline phase. The frequency increases by 0.7 cm^{-1} and 0.5 cm^{-1}, respectively. The increase in the frequency of the methylene symmetric stretching band indicates that the level of acyl chain disorder increases for these samples. There is no change in the level of acyl chain disorder for the liquid crystalline phase for the GCDC containing sample. The GCDC containing sample does, however, show a decrease, of 0.2 cm^{-1}, in the frequency of the methylene symmetric stretching band for the gel phase when compared to the GCDC containing sample hydrated with a solution containing 20 mg/ml ethanol-d_6, indicating an decrease in the level of acyl chain disorder. No change is

observed in the frequency of the gel phase for pure DPPC or the DPPC sample containing GUDC hydrated with a 200 mM KH_2PO_4 solution containing 20 mg/ml ethanol-d_6. Phase transition profiles for DPPC with and without GUDC or GCDC hydrated with a solution containing 120 mg/ml ethanol-d_6 are given in Figure 4.11. The phase transition temperatures increase for all samples and are closer to the phase transition temperature of pure DPPC hydrated with a solution containing no ethanol-d_6. The breadths of the phase transitions for the bile salt containing samples hydrated with a solution containing 120 mg/ml ethanol-d_6 are lower than those obtained for similar samples hydrated with a solution containing 0 or 20 mg/ml ethanol-d_6. The decrease in the breadth of the phase transitions for these samples indicates that the cooperativity between the lipid molecules during the phase transition increases with increasing ethanol concentrations of the hydrating solution. The phase transition parameters for samples hydrated with a 200 mM KH_2PO_4 solution containing 120 mg/ml ethanol-d_6 having an initial pH of 8 are given in Table 4.3.

4.4 Conclusion

The results of the studies presented here show that the effects which the bile salts have on the DPPC bilayer depend on the hydrating conditions, the concentration of ethanol-d_6 in the hydrating solution, and the form of the bile salt (ie., epimeric, conjugated or unconjugated, protonated or unprotonated) present in the sample. The degree to which the bile salts affect the DPPC bilayer is pH dependent for the unconjugated and glycine conjugated forms of UDC and CDC. At higher pH's these bile salts have a greater effect

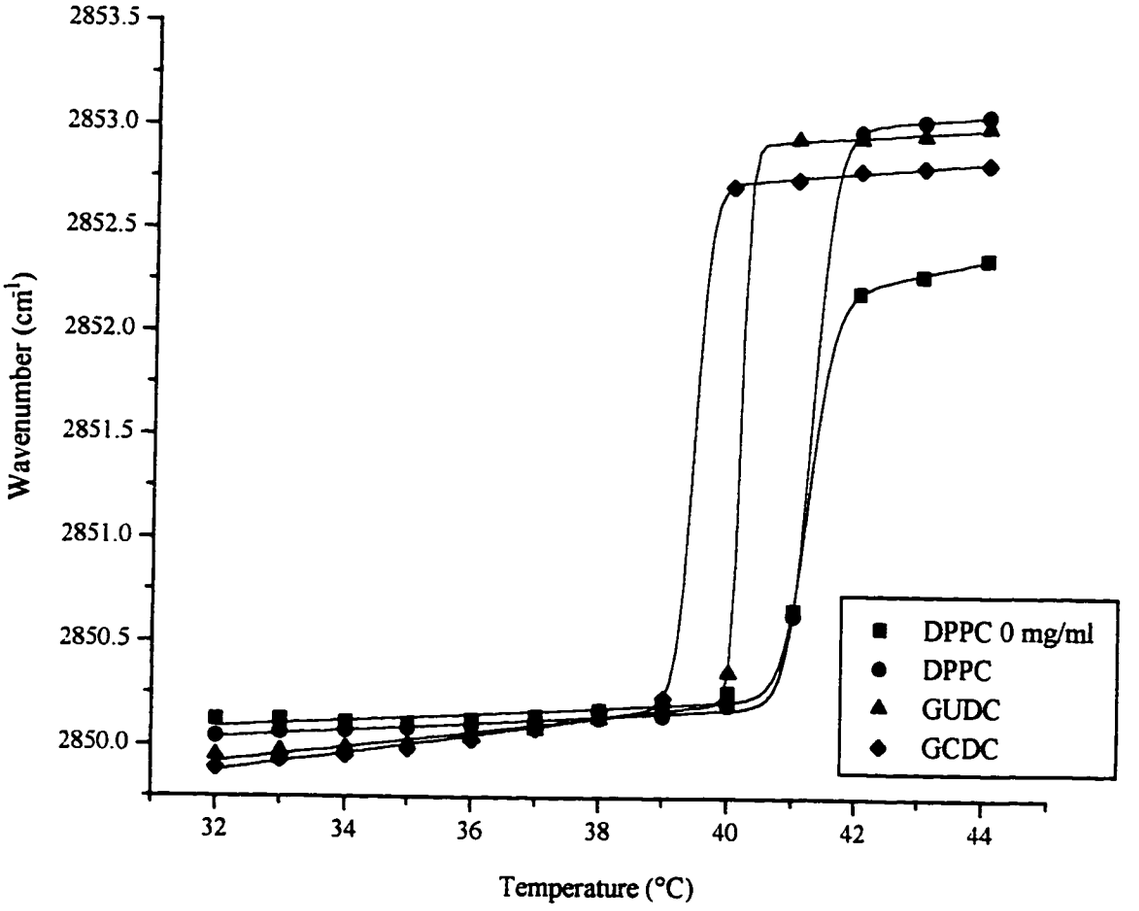

Figure 4.11: Phase Transition Profiles for DPPC/Bile Salt Samples Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 8)

on the breadth of the phase transition (i.e., cooperativity between the lipid molecules of the bilayer during the phase transition), and a lesser effect on the level of acyl chain disorder in the liquid crystalline phase. Differences in the effects which the bile salts have on the DPPC bilayer when hydrated with solutions having different initial pH's may be due to the presence of either the protonated or the unprotonated species of the bile salt being present in the solution. It has been shown that the bile salts have different properties in solutions of different pH.[28] Igimi and Carey have shown that the protonated form of the bile salts are more lipophilic, which likely indicates the protonated bile salts are more likely to partition into the hydrophobic region of the lipid membrane.[28] It is possible that the protonated and the unprotonated forms of the bile salts interact with the lipid assembly in a different manner. At pH's several pH units higher than their pK_a, these compounds are expected to exist primarily in the anion form. The glycine and the unconjugated bile salts have pK_a's approximately equal to 3.8 and 5, respectively, which increases the likelihood of having protonated species at the lower initial pH's studied here.[18, 84] It should be stressed that there are complex acid base and distribution equilibria occurring within the system and the pK_a's of the bile salts have been shown to depend on the concentration of the bile salt.[28]

Arguments for the bile salt interacting with the aqueous interface region of the bilayer stem from studies on lipid bilayers containing cholesterol and studies performed by Bayerl et al. [42] and Guldutuna et al.[44]. Guldutuna et al. studied the interactions of CDC and UDC with red blood cells and hepatocyte membranes using EPR spectroscopy.[44] Guldutuna et al. determined the location of the bile salt by using two

different spin probes to detect the bile salt's position in the lipid bilayer. One probe was used to detect the bile salt in the nonpolar region of the bilayer and a second probe was used for the polar region of the bilayer. The authors concluded that the conjugated form of UDC was located in the polar region of the bilayer and the unconjugated form of UDC was located in the nonpolar region of the bilayer. Bayerl et al. studied the interactions of sodium deoxycholate with DPPC vesicles using FTIR spectroscopy.[42] Bayerl et al. concluded that the FTIR data indicate that the bile salts interact at either of two sites, the aqueous interface region or the acyl chain region, depending on the conditions utilized. They suggest a shift in the phase transition temperature, without significant changes in the number of gauche conformers in either the gel or liquid crystalline phases, indicates an interaction at the aqueous interface region. While significant changes in the frequency of the methylene symmetric stretching band, indicating an ordering of the liquid crystalline phase, provides evidence of the sodium deoxycholate molecules interacting with the second site, the acyl chain region. The basis for their argument stems from a comparison of the data obtained for bile salt samples and others in which cholesterol is incorporated into the DPPC bilayer. Cholesterol is known to interact in the acyl chain region which has been reported to result in an ordering of the liquid crystalline phase, a disordering in the gel phase and an increase in the breadth of the phase transition. The authors suggest that the interaction with the acyl chain region is consistent with an interaction of the sodium deoxycholate at the perimeter of disk shaped micelles.[42]

The location of the bile salt in the lipid assembly will not only depend on the type (unconjugated, taurine conjugated or glycine conjugated) of bile salt present but also on

the form (protonated or unprotonated) of the bile salt present. The conjugated bile salts are more hydrophilic and therefore are more likely to interact with the more polar regions of the lipid bilayer. Results from studies in previous chapters suggest that the unconjugated bile salts interact with aqueous interface region of the lipid bilayer. Similar arguments could be used to postulate the location of the glycine conjugated bile salts in the lipid bilayer. In our studies the effect which the glycine conjugated bile salts have on the bilayer are clearly different from that which has been observed with the incorporation of cholesterol into the DPPC bilayer but similar to results obtained, in previous chapters, for the unconjugated bile salts. The breadth of the phase transition for the glycine conjugated bile salt samples hydrated at higher pH's is consistent with the bile salt inserted into the apolar region of the bilayer, as in the case of cholesterol, but the levels of acyl chain order observed are not consistent with the data available for the models based on the incorporation of cholesterol. The breadth of the phase transition profile decreases with decreasing pH. A result similar to those obtained for the unconjugated bile salts. Since the anion form of the bile salt, which should predominate at higher pH's, is less hydrophobic than the protonated form and, therefore, less likely to interact with the hydrophobic acyl chain region of the bilayer, the bile salt is most likely interacting with the aqueous interface region. These conclusions are similar to those postulated for the location of the unconjugated bile salts in the lipid bilayer in previous chapters.

Once again the most interesting results were obtained for the samples hydrated with solutions containing ethanol-d_6. The presence of ethanol, in the hydrating solutions, results in an increase in the cooperativity between the lipid molecules during the gel to

liquid crystalline phase transition, an increase in the stability of the membrane and an increase in the fluidity of the liquid crystalline phase for the samples containing GUDC and GCDC. Hydrating the lipid assembly with a solution containing 120 mg/ml ethanol-d_6 results in the samples behaving as if there is no bile salt present. Therefore, it can be concluded that ethanol, at high concentrations, subdues the effects which the unconjugated, and glycine conjugated forms of UDC and CDC have on the DPPC bilayer.

The results of several previous studies indicate that ethanol, at high concentrations, interacts with the headgroup.[96, 97] If this is indeed occurring, then the ethanol-d_6 would be occupying the site in the lipid assembly where these bile salts would normally be expected to interact with the lipid assembly. Thus, there may be a competition between the bile salt molecules and the ethanol molecules for this site of interaction. The mole ratio of ethanol to bile salt for DPPC samples containing bile salt hydrated with solutions containing 20 and 120 mg/ml ethanol is approximately 80 and 477, respectively. Thus, the ethanol may be competing more effectively for these sites than the bile salt molecules, thereby, reducing the effects which the bile salt has on the lipid assembly.

Another possible explanation for the limited effect which bile salts have on the lipid bilayer when high concentrations of ethanol are present in the hydrating solution may be due to the fact that ethanol affects the fluidity of the bilayer. The fluidity of the interdigitated gel phase is expected to be less than the fluidity of the "normal" gel phase.[107, 108] The methylene symmetric stretching modes do not appear to be sensitive to the level of fluidity in the gel phase, however, if the gel phase is indeed less fluid, then the bile salt would be expected to have less of an effect on the bilayer. The

latter phenomenon may be due to the fact that bile salts are known to be less disruptive to more ordered bilayers.[109] Thus, the bilayer may be too rigid for the bile salts to affect it since the bile salts may not be able to fit into the location needed to cause disruption of the bilayer.

Since the liquid crystalline phase is more fluid than the gel phase, it is expected that the bile salts would have a greater effect on the liquid crystalline phase of the lipid bilayer. The results presented here show that the fluidity of the liquid crystalline phase increases to approximately the same level for both pure DPPC and the DPPC sample containing bile salt when hydrated with solutions having high ethanol concentrations. Thus, the bile salts appear to be unable to affect the fluidity of the liquid crystalline phase, when hydrating solutions containing high concentrations of ethanol are utilized. The inability to affect the lipid bilayer in the liquid crystalline phase when high ethanol concentrations are present in the hydrating solution may be due to the fact that the bilayer is disordered to its maximum extent. That is, no more gauche conformers can be introduced into the acyl chains since the bilayer has reached its maximum level of acyl chain disorder.

CHAPTER 5

DETERMINATION OF ACYL CHAIN CONFORMATIONAL DISORDER IN AQUEOUS DISPERSIONS OF DPPC/BILE SALT MIXTURES BY MONITORING THE METHYLENE WAGGING MODES

5.1 Introduction

The methylene symmetric and asymmetric stretching bands are used, typically, to monitor changes in the lipid's acyl chains during thermotropic phase transitions. The methylene stretching bands are the strongest and most intense bands in the spectrum of the lipid assembly.[13, 14] The frequencies of the methylene stretching modes increase as the lipid is heated above the gel to liquid crystalline phase transition temperature which is indicative of an increase in the number of gauche conformers in the lipid's acyl chains.[13, 14] Kodati et al. however, warn that caution should be observed when interpreting shifts in the frequencies of the methylene stretching modes as being solely due to changes in conformational order since other possible contributions to the band frequency, including intermolecular coupling and librotorsional motion may occur.[92] Absorption bands due to other modes can also be used to provide information on the conformational order of the lipid's acyl chains. The methylene wagging modes, 1400-1300 cm^{-1}, provide information on the number and type of gauche conformers in the lipid assembly.[69-71, 110] The bands in this region are weak and overlap the strong asymmetric stretching mode of the

phosphate group. A spectrum of this region for pure DPPC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 with the methylene wagging region highlighted is given in Figure 5.1. Typically five bands (1306, 1340, 1355, 1368, and 1378 cm^{-1}) are observed in this region for the lipid assembly. The band at 1378 cm^{-1} is assigned to the methyl group umbrella or symmetric deformation mode.[70, 111] The band has also been called the "methyl symmetric bending (umbrella) mode".[71, 112] This band is typically used as an internal standard since its intensity does not change significantly with changes in acyl chain conformation.[70, 71] The other four bands correspond to the methylene wagging modes. The two bands at 1368 and 1306 cm^{-1} are due to methylene groups which are kinked (k).[70, 71] The band at 1306 cm^{-1} overlaps with the antisymmetric stretching band of the phosphate group of the lipid which is why it is not used typically to provide information on the conformation of the lipid's acyl chains. The bands at 1355 and 1340 cm^{-1} are due to the double gauche (gg) and end gauche (eg) conformers, respectively.[69, 110] The double gauche conformer occurs when there are two adjacent gauche bonds in the hydrocarbon chain, while the end gauche conformer occurs when the terminal methyl group is in a gauche configuration.[111] The wagging modes provide information on the type and number of gauche conformers present in the lipid's acyl chains but do not provide information concerning the location of these particular conformers. If desired, the location of the gauche conformers in the lipid's hydrocarbon chains can be determined by performing experiments with lipids containing deuterated acyl chain segments and monitoring the CD_2 rocking modes.[113, 114]

Casal and McElhaney monitored the wagging bands for a series of

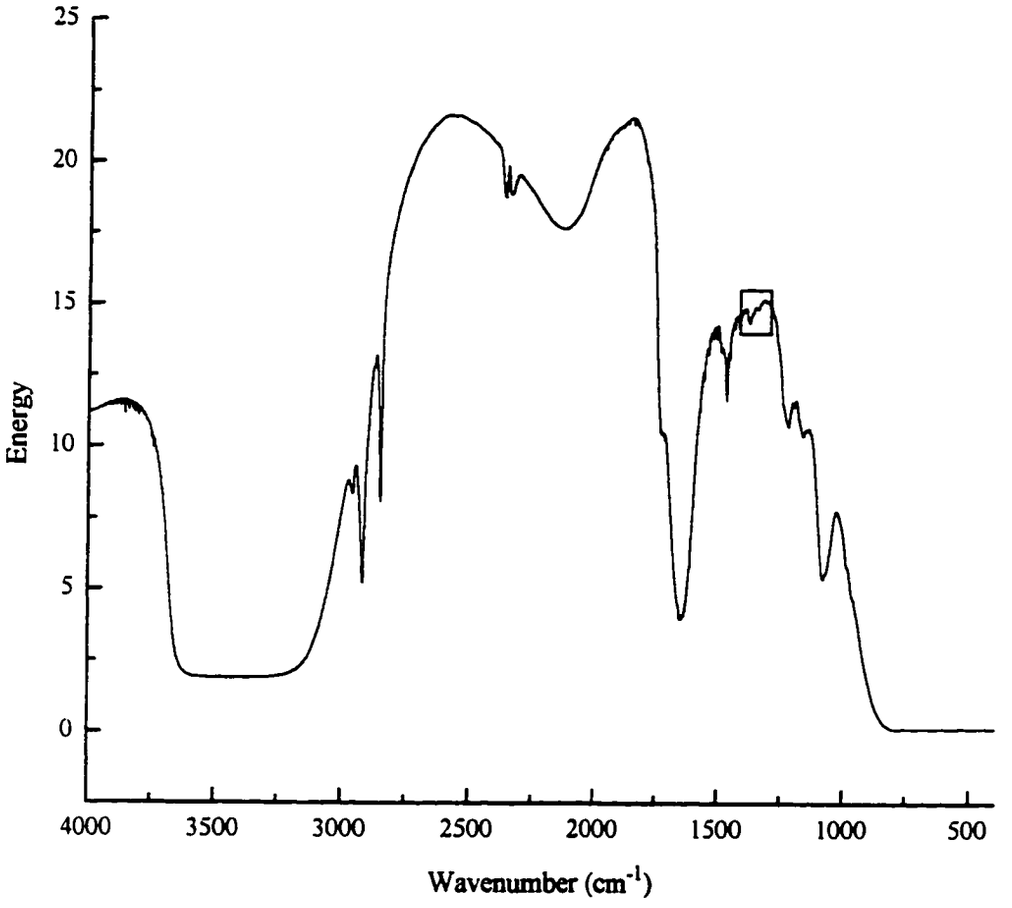

Figure 5.1: Spectrum of Pure DPPC Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7, 32 °C). The methylene wagging modes (~1400 - 1280 cm^{-1} region) are highlighted.

phosphatidylcholines having different length hydrocarbon chains.[71] Studies were performed using multilamellar vesicles with data being acquired for the lipid bilayer in the liquid crystalline phase. The spectra were deconvolved to determine the actual intensity of the wagging modes in the 1400-1300 cm^{-1} region of the infrared spectra. The intensities of the three bands (1367, 1355, 1341 cm^{-1}) were normalized to the intensity of the 1378 cm^{-1} band due to the methyl deformation. The results of this study show the band due to the kink type of conformer has the greatest intensity ratio of the wagging modes. The intensity ratio of this band was found to increase with increasing acyl chain length, thereby implying, longer chain length lipids have more kink conformers in the liquid crystalline phase. The I_{1355}/I_{1378} intensity ratio, which is sensitive to the number of double gauche conformers, increases slightly with increasing chain length while the I_{1340}/I_{1378} intensity ratio, which is sensitive to the number of end gauche conformers, showed no significant change with changes in the length of the hydrocarbon chain. Casal and McElhaney calculated the number of gauche conformers, in the liquid crystalline phase, of DPPC. The calculation involved multiplying the normalized intensities by conversion factors determined by Holler and Callis.[112] Casal and McElhaney determined that there were 1.19 kinks/chain, 0.54 end gauche conformers/chain and 0.40 double gauche conformers/chain for DPPC in the liquid crystalline phase. Based on these calculations, the total number of gauche conformers per chain is equal to 3.72, assuming one gauche conformer in each end gauche and two gauche conformers in each kink or double gauche. These results are within the range (3.6 to 4.2 gauche conformers per chain) of the number of gauche conformers/chain calculated by Mendelsohn et al.[113]

Senak et al. used the methylene wagging modes to determine the number of gauche conformers in two hydrated lipids, 1,2-dipalmitoylphosphatidylethanolamine (DPPE) and DPPC.[70] The results obtained for these lipids were compared to data acquired for long chain alkanes. Multilamellar vesicles of the lipids were used for this study. Data were acquired for the lipid bilayers in the liquid crystalline phase. The spectra were deconvolved, in the 1400-1300 cm^{-1} region to determine band intensities. The results show that the intensities of the bands due to the end gauche and double gauche conformers increase with temperature for the alkanes. The band due to the kink conformers in the alkanes decreased slightly with increasing temperature. Data for multilamellar vesicles of DPPC, in the liquid crystalline phase, show a lower intensity for the band due to the double gauche conformers when compared to the intensity of this band in the spectra acquired for the alkanes. Comparison of the results obtained for DPPC and DPPE showed fewer double gauche (0.2 for DPPE and 0.6 for DPPC at 80 °C) and end gauche (0.2 for DPPE and 0.4 for DPPC at 80 °C) conformers in DPPE than in DPPC. The authors suggest that this is due to the tighter packing of the lipid molecules in DPPE than in DPPC.

The study presented here was performed to determine if changes observed in the frequency of the methylene symmetric stretching modes for the bile salt containing sample are due primarily to the introduction of gauche conformers to the lipid acyl chains or if the shift in frequency is due to other factors. The number and type of gauche conformer introduced into the lipid's acyl chains was also determined. Changes in the intensity of the methylene wagging modes relative to the intensity of the methyl umbrella mode for

samples of pure DPPC and DPPC samples which contain the bile salt, sodium chenodeoxycholate, were monitored. The effects of the initial pH of the hydrating solution and the effect which the presence of ethanol in the hydrating solution have on these modes and thus, the conformation of the lipid's acyl chains, were also determined. The band at approximately 1329 cm^{-1} was also investigated to determine if this band was due to the methylene groups in the headgroup of the lipid.

5.2 Materials and Methods

5.2.1 Materials

Synthetic 1,2 dipalmitoyl-sn-glycero-3-phosphocholine, DPPC, (manufacturer stated purity, > 99%) was purchased, in powder form, from Avanti Polar Lipids, Inc. (Alabaster, AL, lot number 160PC-201). Sodium chenodeoxycholate, CDC, (manufacturer stated purity, > 97%, by TLC) was obtained from Calbiochem (La Jolla, CA). The bile salt was used as received without further purification. Fully deuterated ethanol (ethanol-d_6, manufacturer stated purity, 99+ D %) was purchased from Sigma Chemical Company (St. Louis, MO). Potassium phosphate monobasic (KH_2PO_4) was purchased from Fisher Scientific (Pittsburgh, PA). Anhydrous hexadecane (manufacturer stated purity, 99+%, H_2O <0.005%) was obtained from Aldrich Chemical Company (Milwaukee, WI). Potassium hydroxide (KOH), in pellet form, was obtained from J.T. Baker Chemical Company (Pittsburgh, PA). Laboratory distilled, deionized water was used for the preparation of all solutions.

5.2.2 Sample Preparation

The procedures used to prepare the lipid samples were the same as described in Chapter 3. The lipid samples were hydrated with 200 mM KH_2PO_4 solutions having initial pH's of 5, 7, and 8. Hydrating solutions were also prepared to contain 20 and 120 mg/ml ethanol-d_6 using 200 mM KH_2PO_4, initial pH of 8, as the diluent .

Experiments were also performed to determine the spectral contribution of the bile salt to the intensities of the methylene wagging modes. Solutions containing sodium chenodeoxycholate at concentrations of 0.0, 4.6, 12.1 and 29.0 mM were used for this experiment. The solutions were prepared by dissolving the bile salt in an appropriate volume of 200 mM KH_2PO_4 stock solution which had been previously adjusted to a pH of 7.

5.2.3 Data Acquisition

The data were acquired and analyzed as described in Chapter 2 with the following modifications. Due to the inherent weakness of the methylene wagging modes, the pathlength employed for these samples was increased to 25 microns. Twenty microliter aliquots of the sample (either hydrated lipid or neat hexadecane) were placed between two CaF_2 (Spectral Systems, Hopewell Junction, NY) windows. Single beam (ie., energy) spectra were converted to absorbance spectra by ratioing to an air background. The individual absorption profiles for the bands at 1340, 1355, 1368, and 1378 cm^{-1} were determined by fitting the absorbance profile for the methylene wagging region (1320-1400 cm^{-1}) using the Peak Fitting Module of the Origin package (Microcal Software, Inc.,

Northampton, MA). It was found that the best fits of the bands could be obtained using either Lorentzian or modified Gaussian peak shapes. Initial determination of the positions of the bands was based on values obtained from the literature indicating that the band indicative of the kink conformer is located at 1368 cm^{-1}, the band indicative of the double gauche conformer at 1355 cm^{-1} and the band indicative of the end gauche conformer at 1340 cm^{-1}. These values are consistent with those obtained from Fourier Self Deconvolution (FSD) of the bands in the methylene wagging modes. FSD was performed for selected samples using a program provided with the OMNIC software (Nicolet Analytical Instruments, Inc., Madison, WI). Prior to fitting the absorption profiles the baseline was corrected, between 1400 and 1320 cm^{-1}, using a spline function. The best results were obtained when the maxima of the bands, at 1368 cm^{-1} and 1341 cm^{-1}, corresponding to the kink and end gauche conformers, and the width of the peak at ~1340 cm^{-1}, corresponding to the end gauche conformer, were allowed to vary during the fitting process. The band corresponding to the double gauche conformer (1355 cm^{-1}) is not evident in the spectrum for the gel phase of the lipid bilayer. Therefore, this band was only fit for the lipid in the liquid crystalline phase, where there was evidence of this band in the spectrum. The widths of the absorption bands and the positions of the peak maxima were maintained during the fit for the band at 1355 cm^{-1}, which has been assigned to the double gauche conformer. The fitting process was repeated until the value of Chi squared (a measurement of the difference between the actual and the expected data) remained constant. The intensities of the deconvolved methylene wagging bands were ratioed to the intensity of the deconvolved methyl umbrella band. The intensity ratios of the bands were

plotted versus temperature to determine the effect which the addition of CDC, the pH of the hydrating solution and the addition of ethanol to the hydrating solution have on the number and type of gauche conformers in the lipid's acyl chains in both the gel and liquid crystalline phases of the DPPC bilayer assembly.

The data for the experiments performed to determine the spectral contribution of the bile salt to the intensities of the methylene wagging modes were obtained in the following manner. Spectra were first acquired for a DPPC sample hydrated with 200 mM KH_2PO_4 solution at an initial pH of 7 at two temperatures, 32 and 44 °C. These temperatures were chosen to provide information on the lipid bilayer in the gel and the liquid crystalline phases. Once the spectra for DPPC were acquired, a sodium chenodeoxycholate solution was placed in a standard liquid cell consisting of two CaF_2 windows separated by a 25 μm PTFE spacer. The cell was placed on the source side, directly in front of the thermoelectrically heated stage containing the DPPC sample in the sample compartment. Spectra were then simultaneously acquired for the bile salt solution, at room temperature, and the DPPC sample at temperatures of 32 and 44 °C. After acquiring spectra for the DPPC sample with each of the CDC solutions, individual spectra for each of the bile salt solutions were obtained. Data were acquired using the same procedure for each of the concentrations of CDC studied. The DPPC sample was never removed from the stage, but the position of the stage was adjusted to maximize the amount of light reaching the detector for each CDC solution.

5.3 Results and Discussion

5.3.1 Experiment to Determine the Magnitude of the Spectral Contribution of the Bile Salt in the Methylene Wagging Region

The goal of this work was to utilize the intensities of the methylene wagging modes to indicate how the number and type of gauche conformers in the lipid's acyl chains are affected by the presence of bile salts in the lipid bilayer. It is, therefore, important to establish the effects which the presence of the added bile salts have on the intensities determined for the methylene wagging bands of the lipid assembly due to spectral interferences. A typical absorbance spectrum in the methylene wagging region for pure DPPC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 is shown in Figure 5.2. The deconvolved peaks are also shown in this figure.

Several CDC containing solutions were used to evaluate the spectral contribution of the bile salts to the intensities of the methylene wagging band of the lipid assembly. In order to compare the data obtained for studies which use bile salts at a concentration of 15 mole percent (85 mole % lipid) to the data obtained here, bile salt solution concentrations must be converted to an equivalent concentration corresponding to the mole percent bile salt in the lipid sample. The calculations used are similar to the calculation described in Chapter 2 for the evaluation of the spectral contribution of the bile salt to the frequency of the methylene symmetric stretching band.

To evaluate the magnitude of the effect which the bile salts have on the intensity ratios for the methylene wagging modes, the differences between the intensity ratios obtained for pure, hydrated DPPC with a KH_2PO_4 solution (sample arrangement A) and

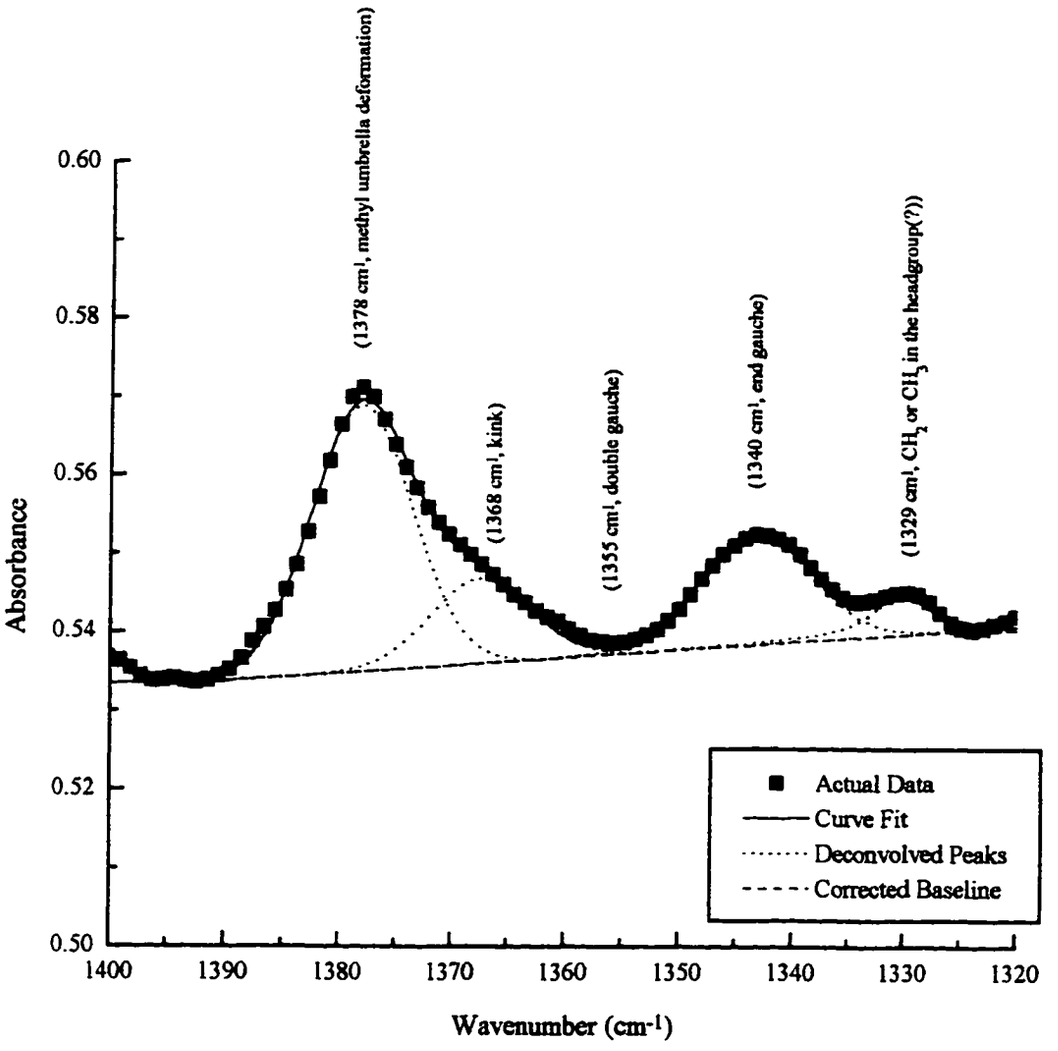

Figure 5.2: Absorbance Spectrum of Pure DPPC Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7, 32 °C)

pure, hydrated DPPC with the several CDC solutions (sample arrangement B) were determined. For clarity, this difference will be called "difference 1".

$$\text{difference 1} = (I_{1368,\,1340}/I_{1378})_B - (I_{1368,\,1340}/I_{1378})_A \qquad (1).$$

The calculated differences are given in Table 5.1 and plots of the calculated differences versus the equivalent CDC concentrations for the kink and end gauche conformers are presented in Figures 5.3 and 5.4, respectively. The differences in the intensity ratio for the kink conformers increase with increasing CDC concentration for the bilayer in the gel phase while the differences in the intensity ratios for the kink conformers in the liquid crystalline phase are similar for all CDC concentrations studied. The differences in the intensity ratios corresponding to the end gauche conformers decrease with an increase in the CDC concentration for the lipid bilayer in both the gel and liquid crystalline phases.

The differences between the I_{1368}/I_{1378} intensity ratios obtained for pure DPPC and a KH_2PO_4 solution and DPPC with a CDC solution having an equivalent concentrations of 15 mole percent were determined to be +0.006 and +0.021 in the gel and liquid crystalline phases, respectively corresponding to percent differences of 1.75% and 5.80%, respectively. Differences for the I_{1340}/I_{1378} intensity ratios for the same samples were -0.003 and -0.011 for the gel and liquid crystalline phases which are equivalent to percent differences of -0.78 and -4.87%, respectively. Thus, differences in the I_{1368}/I_{1378} intensity ratio corresponding to the kink conformers determined between pure DPPC and the DPPC samples containing 15 mole percent CDC which are greater than 0.006 in the gel phase and greater than 0.021 in the liquid crystalline phases indicate that CDC is causing changes in the number of kink conformers in the acyl chains.

Table 5.1: Differences and Percent Differences Utilized in the Determination of the Spectral Contribution of CDC to the Methylene Wagging Modes

			Delta*		Percent Difference (%)**	
CDC Conc. (mM)	Equiv. CDC Conc. (mole %)	T (°C)	Kink	End Gauche	Kink	End Gauche
4.6	6.5	32	-0.014	+0.019	-4.08	4.96
		44	+0.031	+0.006	8.56	2.65
12.1	14.8	32	+0.006	-0.003	1.75	-0.78
		44	+0.021	-0.011	5.80	-4.87
29.0	29.4	32	+0.019	-0.069	5.54	-18.0
		44	+0.031	-0.050	8.56	-22.1

* Delta = $(I_{1368,\ 1340}/I_{1378})_{\text{Pure DPPC w/CDC Soltn.}} - (I1_{368,\ 1340}/I_{1378})_{\text{Pure DPPC w/ KH2PO4 Soltn.}}$

** Percent Difference = $[[(I_{1368,\ 1340}/I_{1378})_{\text{Pure DPPC w/CDC Soltn.}} - (I1_{368,\ 1340}/I_{1378})_{\text{Pure DPPC w/ KH2PO4 Soltn.}}]/(I1_{368,\ 1340}/I_{1378})_{\text{Pure DPPC w/ KH2PO4 Soltn.}}] * 100$

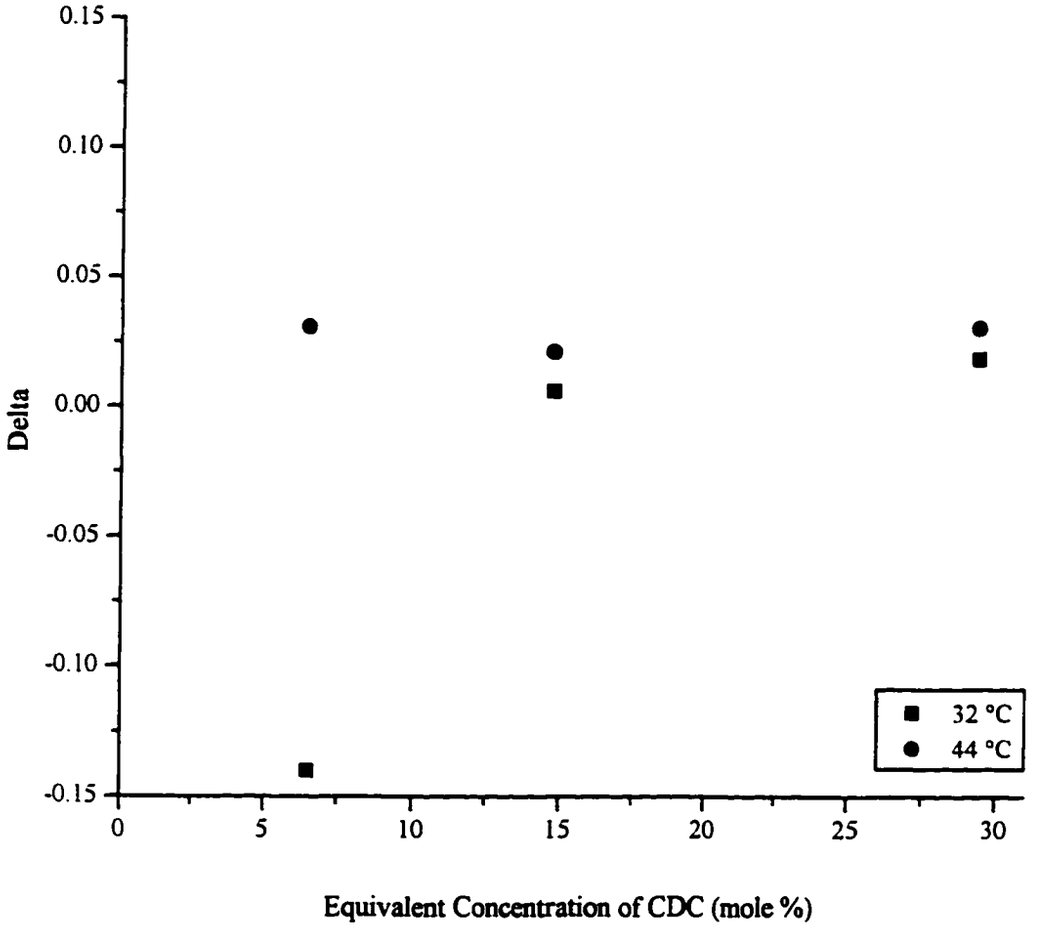

Figure 5.3: Plot of Delta for the Kink Conformers Versus Equivalent Concentration of CDC in a 200 mM KH_2PO_4 Solution (Initial pH = 7)

$$\text{Delta} = [(I_{1368}/I_{1378})_{\text{Pure DPPC w/ CDC Soltn.}}] - [(I_{1368}/I_{1378})_{\text{Pure DPPC w/ KH2PO4 Soltn.}}]$$

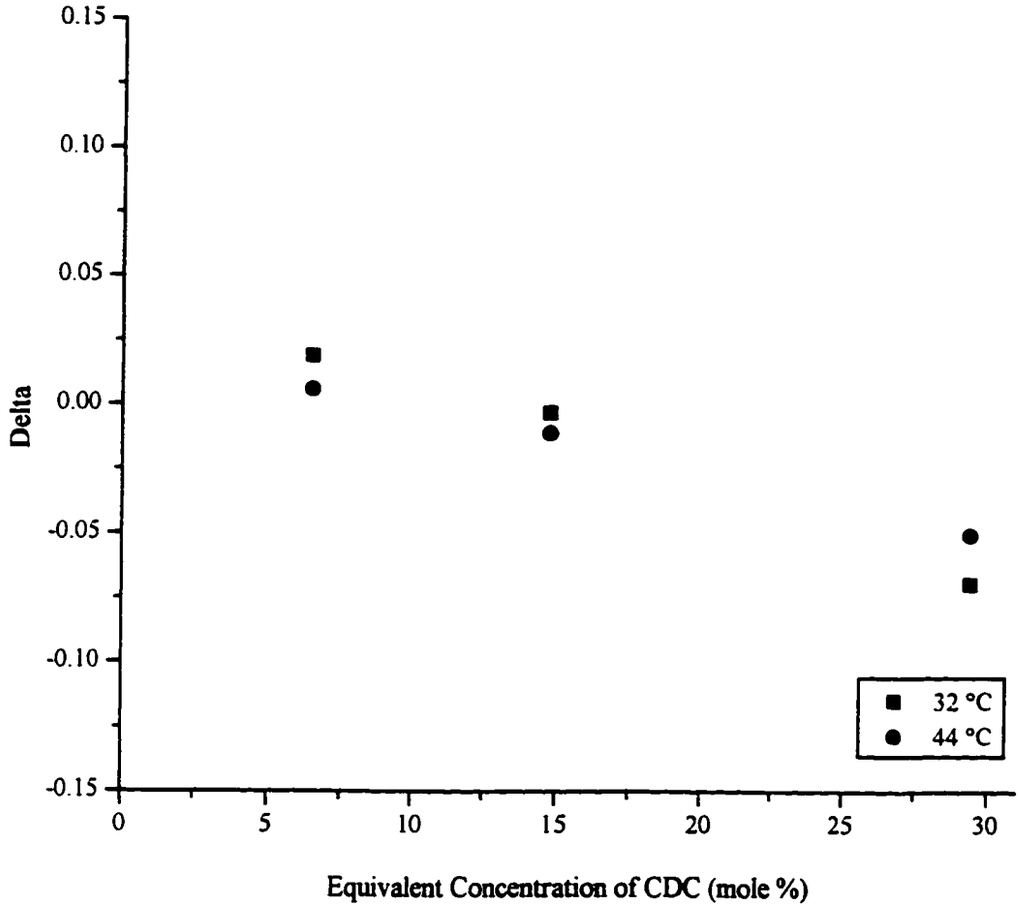

Figure 5.4: Plot of Delta for the End Gauche Conformers Versus Equivalent Concentration of CDC in a 200 mM KH_2PO_4 Solution (Initial pH = 7)

$$\text{Delta} = [(I_{1368}/I_{1378})_{\text{Pure DPPC w/ CDC Soltn.}}] - [(I_{1368}/I_{1378})_{\text{Pure DPPC w/ KH2PO4 Soltn.}}]$$

5.3.2 Effect of CDC on the Type and Number of Gauche Conformers in the Lipid's Acyl Chains for Samples Hydrated with 200 mM KH_2PO_4 Solutions Having Initial pH's of 5, 7, or 8

Experiments were performed to determine the effect which the presence of 15 mole percent CDC has on the number and type of gauche conformers present in the lipid's acyl chains for a DPPC multilamellar assembly hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 . A plot of the I_{1368}/I_{1378} intensity ratio versus temperature for pure DPPC and DPPC containing 15 mole percent CDC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 is given in Figure 5.5. The presence of CDC results in an increase in the I_{1368}/I_{1378} intensity ratio for both the gel and liquid crystalline phases. The I_{1368}/I_{1378} intensity ratio increases by ~0.05 for the gel phase and by ~0.02 in the liquid crystalline phase when compared to pure DPPC hydrated under similar conditions. The differences in the intensity ratios correspond to a 14.0% difference in the gel phase and only a 5.0% difference in the liquid crystalline phase. This suggests that there is an increase in the number of kink conformers in the gel phase due to the presence of CDC. Since the differences determined for the liquid crystalline phase is approximately the same as the difference determined in the previous experiment (which showed that the spectral contribution of CDC caused a difference of 0.02 in the I_{1368}/I_{1378} intensity ratio), CDC does not appear to affect significantly the number of kink conformers in the liquid crystalline phase. A summary of the intensity ratios at 32 and 44 °C for these samples is given in Table 5.2. The phase transition profiles obtained using the I_{1368}/I_{1378} intensity ratio indicate that the phase transition for pure DPPC is sharper than the phase transition profile

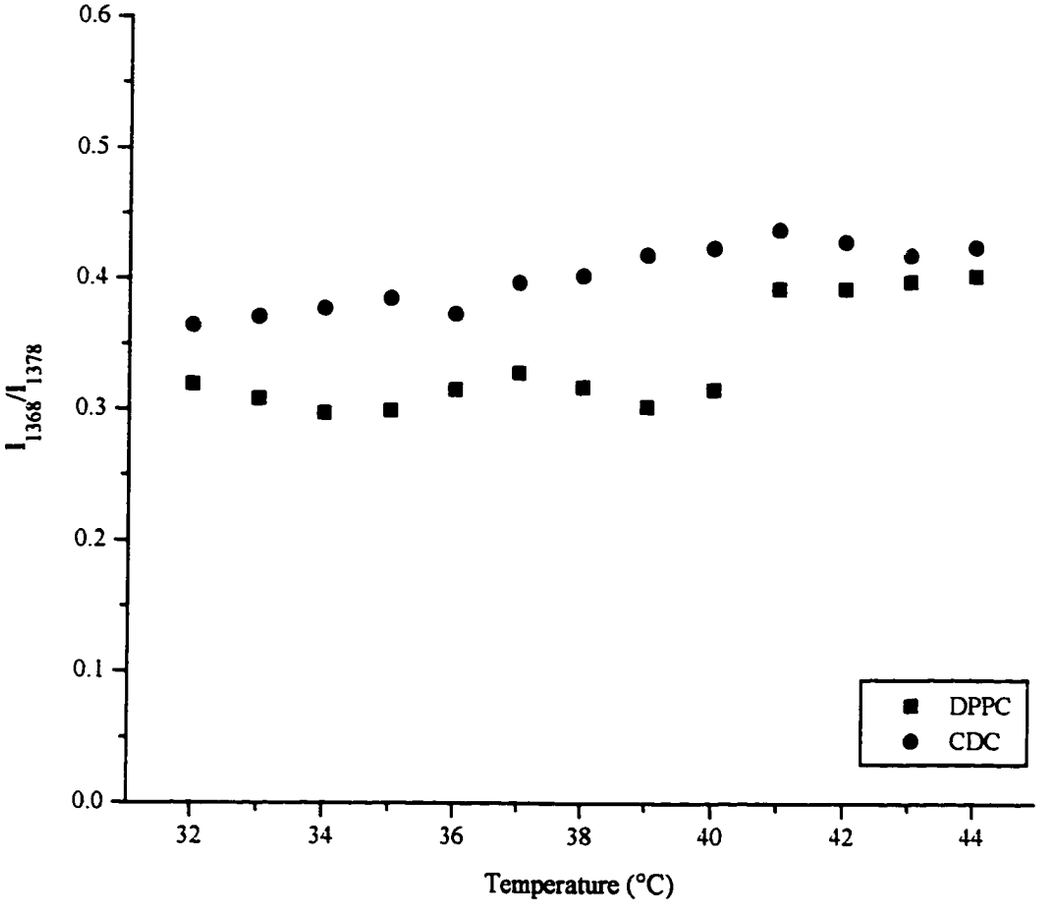

Figure 5.5: Phase Transition Profiles for the Kink Conformers of DPPC/CDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7)

Table 5.2: Intensity Ratios for the Kink (I_{1368}/I_{1378}) and End Gauche (I_{1340}/I_{1378}) Conformers of Pure DPPC and DPPC/CDC Samples for Several Hydrating Conditions

		T (°C)	I_{1368}/I_{1378}	I_{1340}/I_{1378}
pH 7				
	DPPC	32	0.319	0.408
		44	0.402	0.221
	DPPC/CDC	32	0.364	0.274
		44	0.424	0.138
pH 5				
	DPPC	32	0.288	0.396
		44	0.400	0.204
	DPPC/CDC	32	0.375	0.281
		44	0.442	0.131
pH 8				
	DPPC	32	0.310	0.433
		44	0.354	0.200
	DPPC/CDC	32	0.358	0.305
		44	0.457	0.190
pH 8/20 mg/ml Ethanol-d_6				
	DPPC	32	0.308	0.339
		44	0.420	0.206
	DPPC/CDC	32	0.361	0.319
		44	0.445	0.176
pH 8/120 mg/ml Ethanol-d_6				
	DPPC	32	0.351	0.205
		44	0.502	0.150
	DPPC/CDC	32	0.420	0.251
		44	0.541	0.154

obtained for the CDC containing sample. The gel to liquid crystalline phase transition occurs at a lower temperature for the CDC containing sample than for pure DPPC. These results are similar to those based on the methylene symmetric stretching modes for similar samples (Chapter 3).

The effect which CDC has on the number of end gauche conformers was determined using the I_{1340}/I_{1378} intensity ratio. A plot of the I_{1340}/I_{1378} intensity ratio versus temperature is given in Figure 5.6. The I_{1340}/I_{1378} intensity ratio for the sample containing CDC decreases by 0.13 in the gel phase and by 0.08 in the liquid crystalline phase when compared to pure DPPC. These differences correspond to a -32.8 and -37.6% difference in the I_{1340}/I_{1378} intensity ratio for the end gauche conformers in the gel and liquid crystalline phases, respectively. Thus, the data suggest that the presence of CDC in the lipid bilayer decreases the number of end gauche conformers in the lipid's acyl chains. Intensity ratios for these samples at 32 and 44 °C are given in Table 5.2 for comparison. The results for the number of end gauche conformers are different than the data found in the literature.[70] Thus, further studies are necessary to determine why there are discrepancies between the two sets of data. The phase transition profiles for the end gauche conformers show a sharper phase transition and a higher phase transition temperature for pure DPPC than for the DPPC sample containing CDC. Similar results were observed for in the phase transition profiles obtained using the kink conformers and when the methylene symmetric stretching bands were monitored.

Data were also acquired using a hydrating solution having a lower initial pH to determine if decreasing the pH of the hydrating solution also affected the number and type

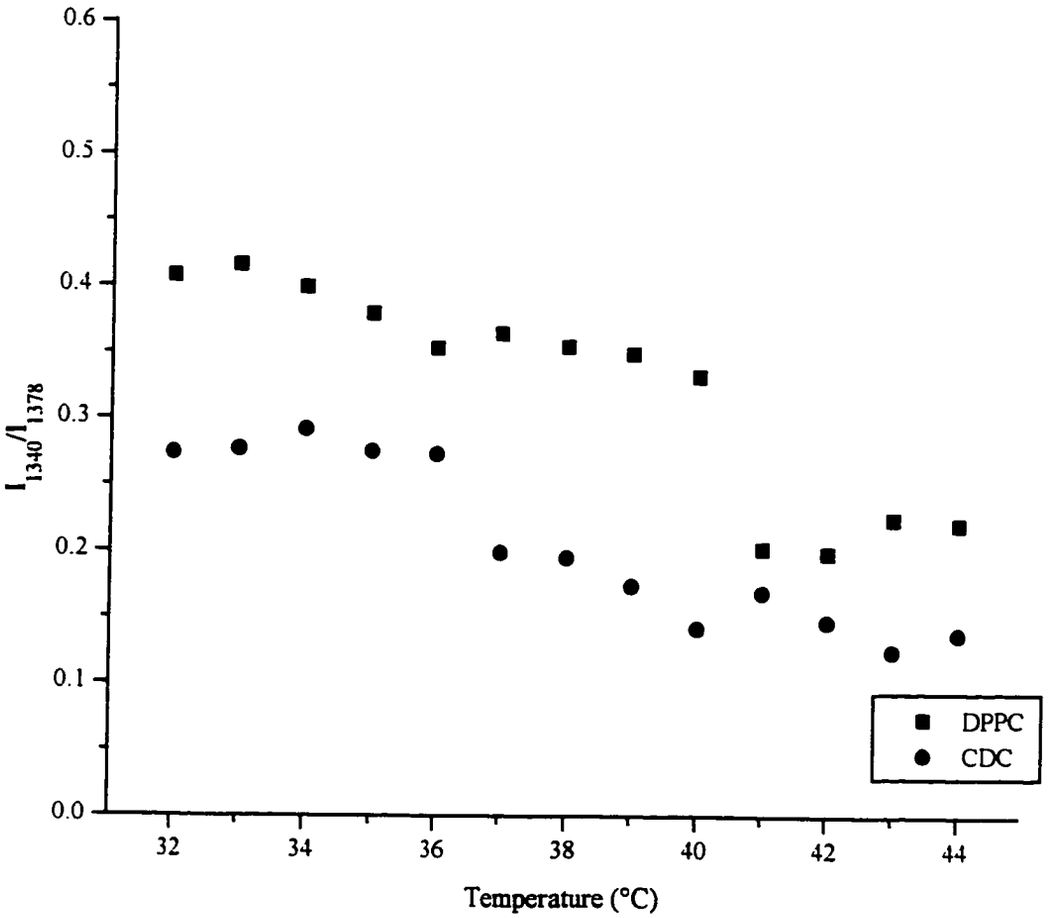

Figure 5.6: Phase Transition Profiles for the End Gauche Conformers of DPPC/CDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7)

of gauche bonds present in the lipid's acyl chains for pure DPPC and CDC containing samples. A plot of the I_{1368}/I_{1378} intensity ratio versus temperature for pure DPPC and DPPC containing CDC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 5 is given in Figure 5.7. The I_{1368}/I_{1378} intensity ratio is greater at 32 °C and 44 °C for the sample which contains CDC when compared to the I_{1368}/I_{1378} intensity ratio obtained for pure DPPC. The intensity ratio increases by 0.09 or 30.2 % for the bilayer in the gel phase and by 0.04 or 10.5 % for the bilayer in the liquid crystalline phase. The percent differences determined between the CDC containing sample and pure DPPC are large suggesting that CDC causes an increase in the number of kink conformers in both the gel and liquid crystalline phases. The percent difference in the gel phase is larger than that determined for the liquid crystalline phase indicating that CDC affects the number of kink conformers in the gel phase to a greater extent than it does in the liquid crystalline phase. The results obtained for the samples hydrated with a solution having an initial pH of 5 are similar to the results obtained for similar samples hydrated with a solution having an initial pH of 7. The phase transition profiles based on the number of kink conformers in the acyl chains indicate that the phase transition occurs at a lower temperature and the cooperativity between the lipid molecules is lower for the CDC containing sample in comparison to pure DPPC. These results are consistent with those obtained for the methylene symmetric stretching band.

A plot of I_{1340}/I_{1378} intensity ratio versus temperature which is given in Figure 5.8, shows the effect which decreasing the initial pH of the hydrating solution has on the number of end gauche conformers in the lipid acyl chains for pure DPPC and DPPC with

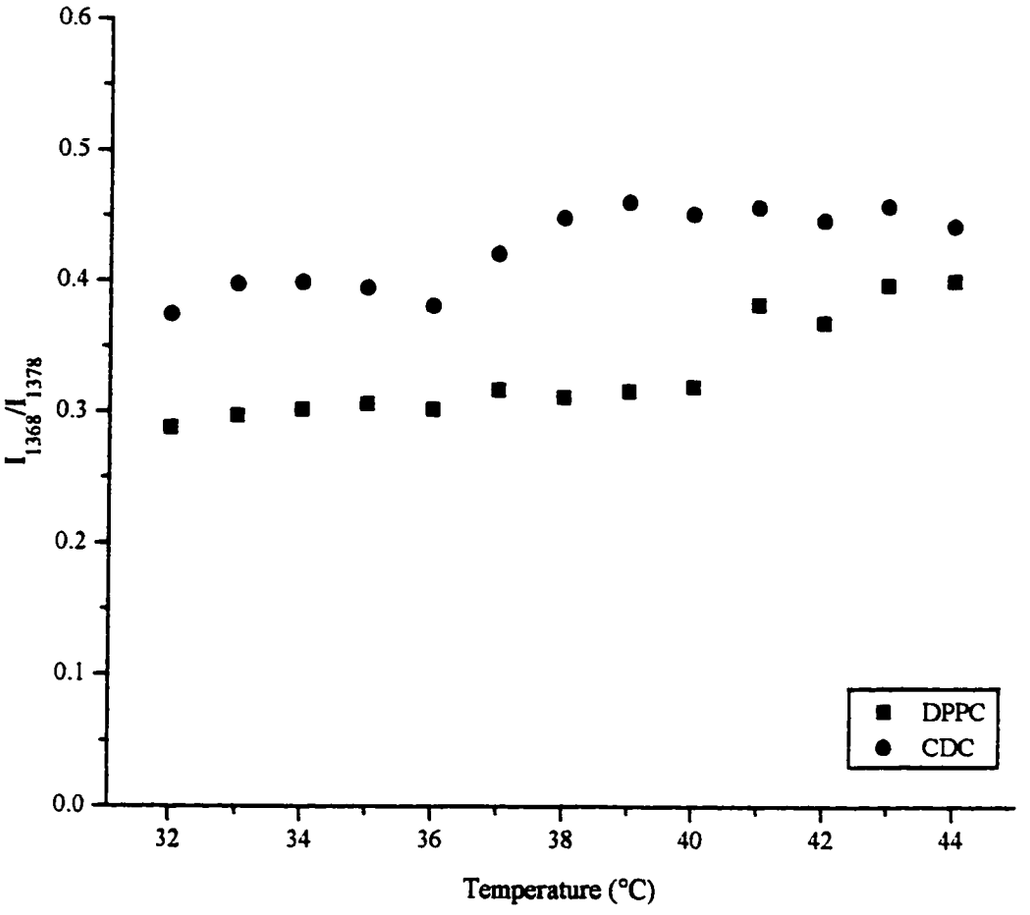

Figure 5.7: Phase Transition Profiles for the Kink Conformers of DPPC/CDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 5)

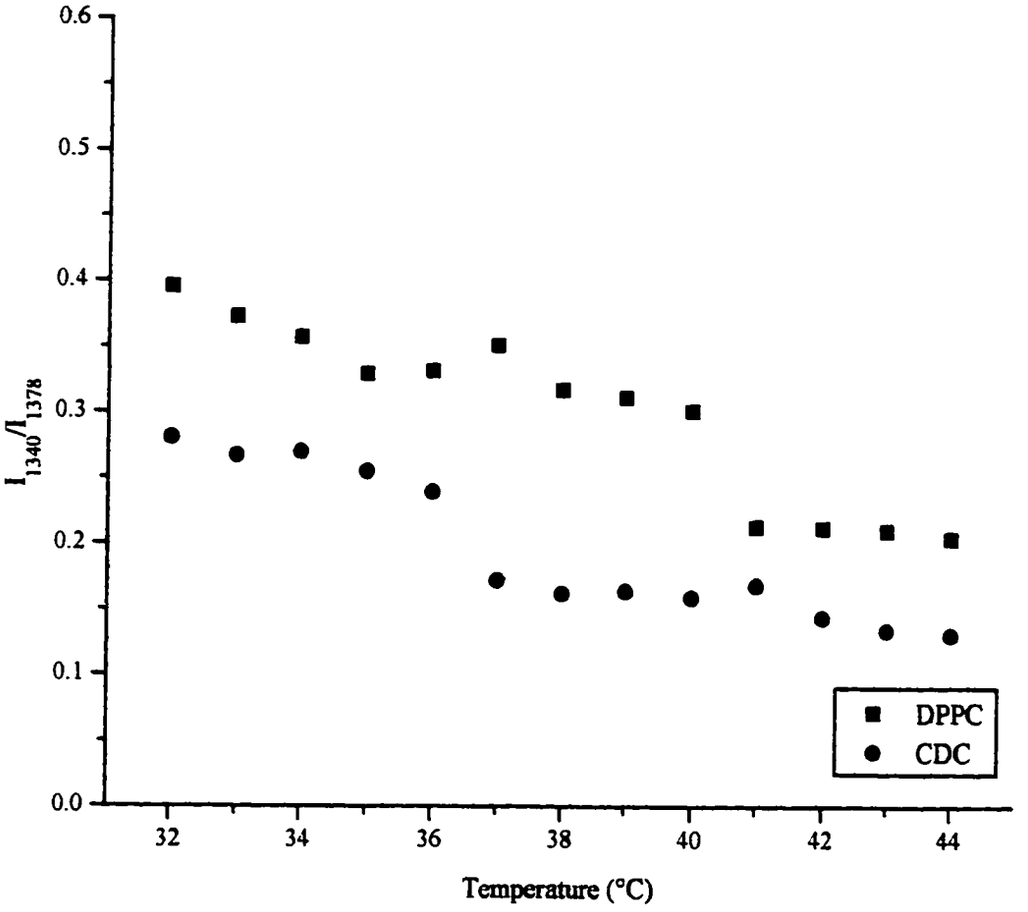

Figure 5.8: Phase Transition Profiles for the End Gauche Conformers of DPPC/CDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 5)

15 mole percent CDC. The I_{1340}/I_{1378} intensity ratio decreases by 0.12 in the gel phase and by 0.07 in the liquid crystalline phase for the CDC containing sample compared to the pure DPPC sample. The differences in the intensity ratios determined between pure DPPC and DPPC containing 15 mole percent CDC correspond to percent differences of -29.0 and -35.8% for the gel and liquid crystalline phases, respectively, indicating that the presence of CDC results in an decrease in the number of end gauche conformers in the lipid's hydrocarbon chains. The values of the intensity ratios for the end gauche conformers are given in Table 5.2. The phase transition for the CDC containing sample occurs at a lower temperature and the cooperativity of the gel to liquid crystalline phase is lower for this sample than for pure DPPC as indicated by the number of end gauche conformers per acyl chain. Similar results were obtained when analyzing the phase transition profiles for the kink conformers and when monitoring the methylene symmetric stretching modes.

Experiments were also performed to determine the effect which increasing the initial pH of the hydrating solution has on the interaction of the bile salts with the DPPC bilayer. The I_{1368}/I_{1378} intensity ratio increased for both the gel and the liquid crystalline phases of the CDC containing samples hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 8 when compared to pure DPPC hydrated with a similar solution. A plot of the I_{1368}/I_{1378} intensity ratio versus temperature is given in Figure 5.9 for pure DPPC and DPPC with 15 mole percent CDC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 8. For the CDC containing sample, the I_{1368}/I_{1378} intensity ratio increases by 0.05 in the gel phase corresponding to a percent difference of 15.5% and by 0.10 in the

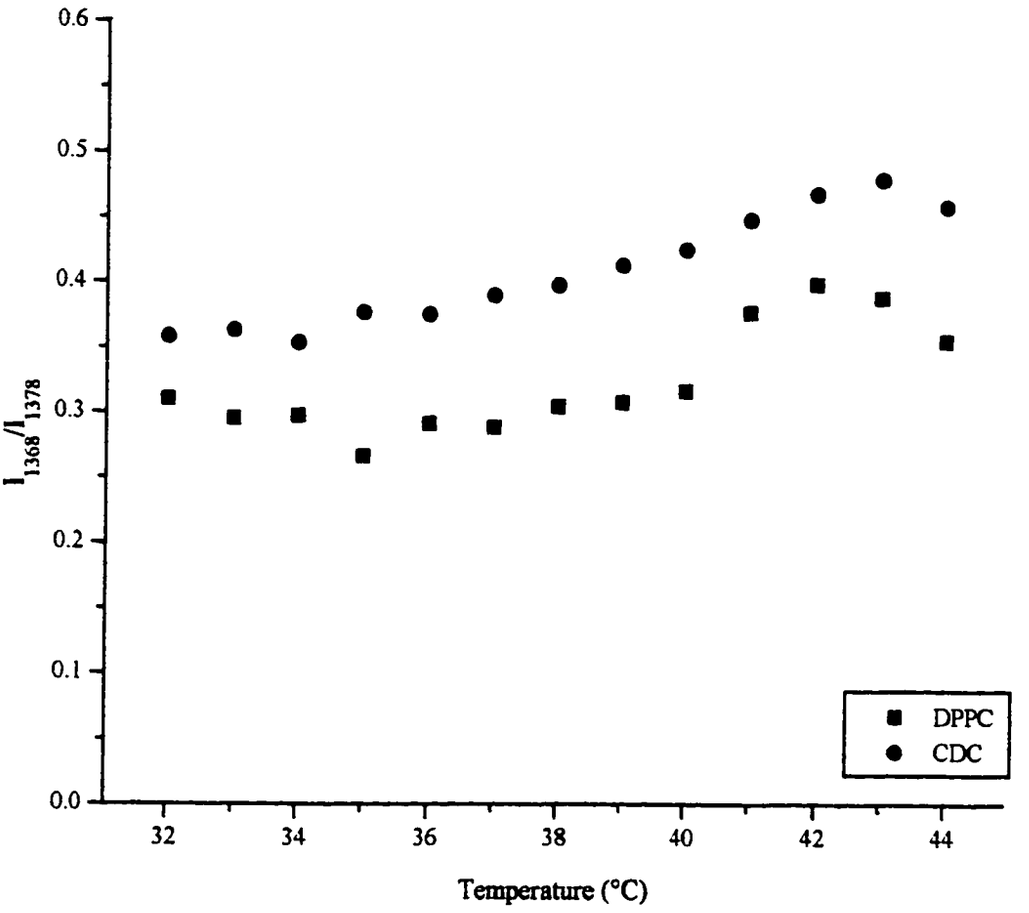

Figure 5.9: Phase Transition Profiles for the Kink Conformers of DPPC/CDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 8)

liquid crystalline phase which is equivalent to a percent difference of 29.1%. The increase in the I_{1368}/I_{1378} intensity ratio and the large percent differences indicate an increase in the number of kink conformers in the lipid's acyl chains due to the presence of CDC in the lipid bilayer in both the gel and liquid crystalline phases. Intensity ratios for these samples are summarized in Table 5.2. The phase transition profiles based on the number of kink conformers per acyl chain indicate that both the phase transition temperature and the cooperativity between the lipid molecules during the phase transition are lower for the CDC containing sample than for pure DPPC. These results are once again consistent with those obtained for the methylene symmetric stretching bands.

The I_{1340}/I_{1378} intensity ratio decreases by 0.13 in the gel phase and by 0.01 in the liquid crystalline phase for the CDC containing sample compared with the pure DPPC sample. This decrease in the I_{1340}/I_{1378} intensity ratio in the gel phase indicates a decrease in the number of end gauche conformers for the CDC containing samples while the number of end gauche conformers in the liquid crystalline phase is approximately the same. A plot of the I_{1340}/I_{1378} intensity ratio versus temperature is given in Figure 5.10 and a summary of the intensity ratios is given in Table 5.2 for pure DPPC and DPPC containing 15 mole percent CDC hydrated with a solution having an initial pH of 8. The phase transition profile for the CDC containing sample based on the number of end gauche conformers is very broad indicating that the cooperativity of the lipid molecules during the phase transition is less than the cooperativity of the lipid molecules during the phase transition for pure DPPC a result which is consistent with those obtained when the methylene symmetric stretching modes were monitored. The methylene symmetric

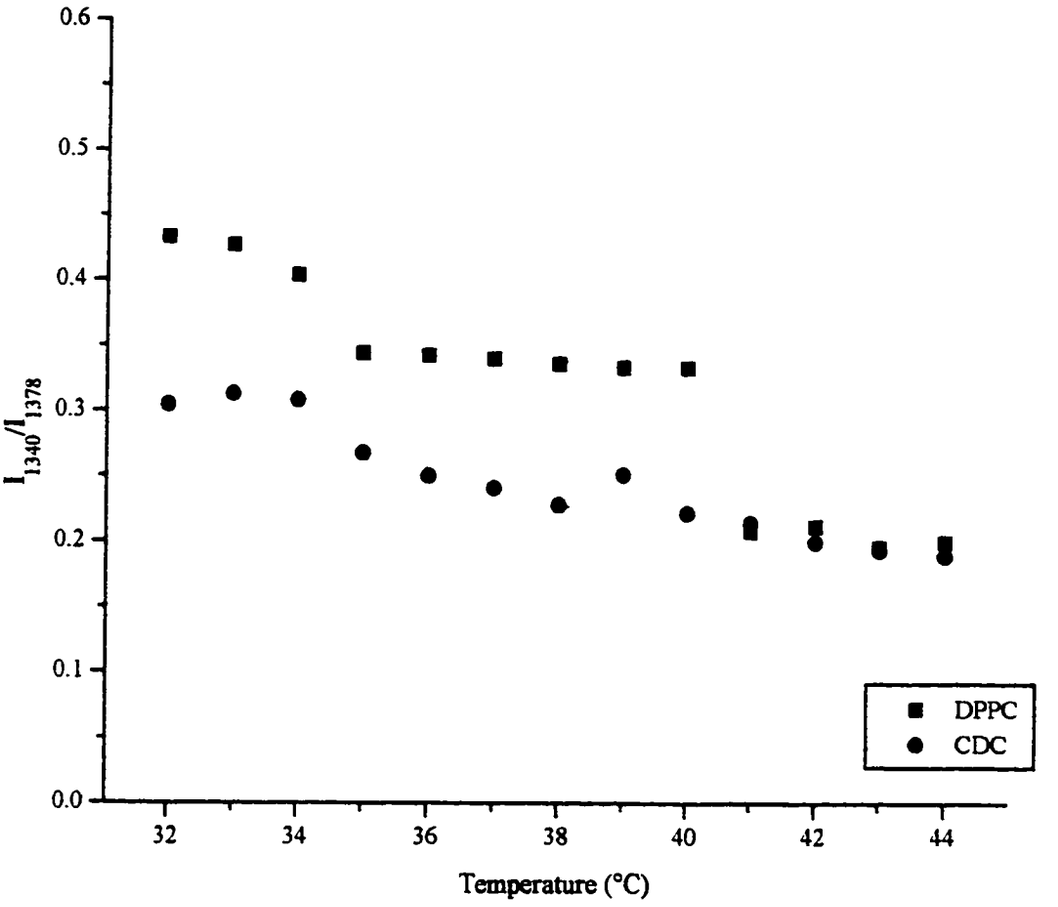

Figure 5.10: Phase Transition Profiles for the End Gauche Conformers of DPPC/CDC Samples Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 8)

stretching mode data are presented in Chapter 3.

Comparison of the data obtained shows that the pH of the hydrating solution does not significantly affect the number of kink conformers in the hydrocarbon chains of the pure DPPC samples for hydrating solutions having initial pH's of 5, 7, and 8. Plots of the I_{1368}/I_{1378} intensity ratios versus temperature for pure DPPC hydrated with 200 mM KH_2PO_4 solutions having initial pH's of 5, 7, and 8 are presented in Figure 5.11. It should be noted that there are some differences but they are likely to be due to errors associated with fitting the spectral data. This data is consistent with the results determined when the methylene symmetric stretching mode was monitored.

Plots of the I_{1368}/I_{1378} intensity ratio versus temperature for DPPC samples containing CDC hydrated with solutions having initial pH's of 5, 7, and 8 are given in Figure 5.12. The number of kink conformers present in the CDC containing samples hydrated with solutions having initial pH's of 7 and 8 is lower in the gel phase when compared to similar samples hydrated with a solution having an initial pH of 5. No trends were observed in the number of kink conformers in the liquid crystalline phase for samples hydrated with solutions having different initial pH's. The phase transition profile for the CDC containing sample hydrated with a solution having an initial pH of 5 appears to be slightly sharper than the phase transition profiles obtained for similar samples hydrated with solutions having initial pH's of 7 or 8. These results differ slightly from those obtained from monitoring the methylene symmetric stretching modes. The results from the methylene stretching mode data, presented in Chapter 3, indicate that there are no changes in the number of gauche conformers in the gel phase for CDC containing samples

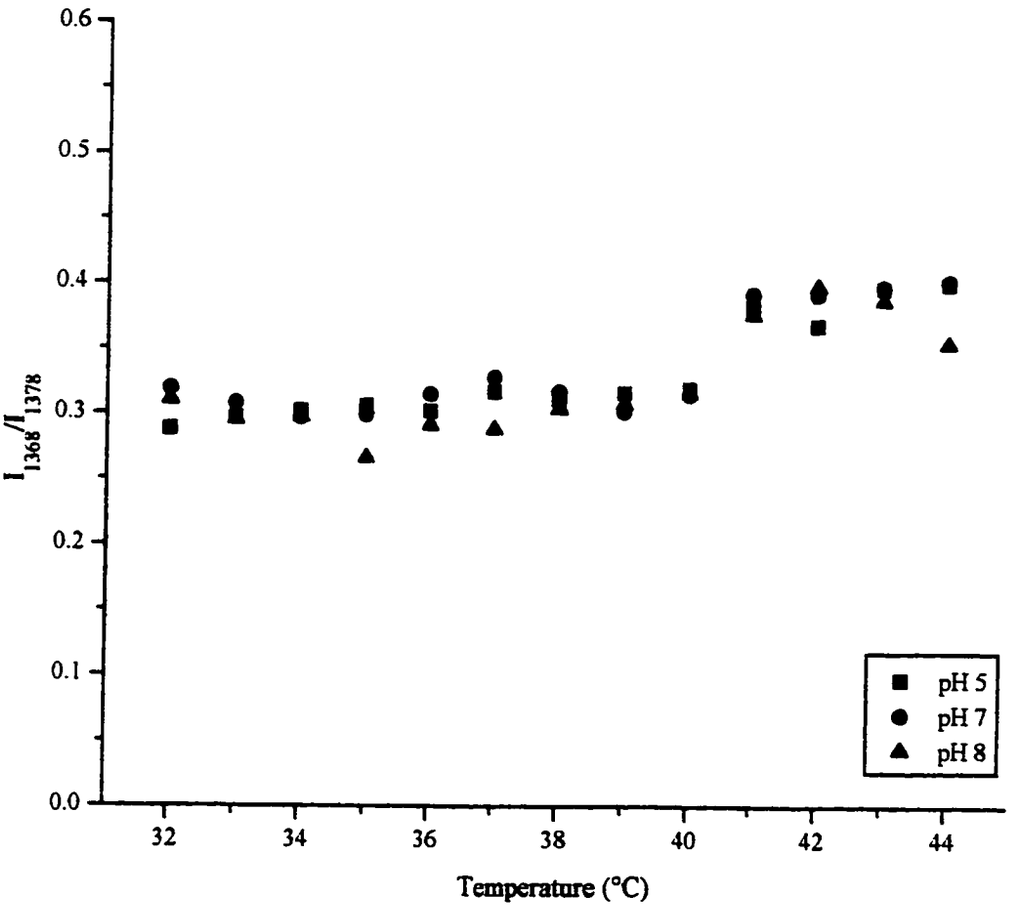

Figure 5.11: Phase Transition Profiles for the Kink Conformers of Pure DPPC Samples Hydrated with 200 mM KH_2PO_4 Solutions at Various Initial pH's

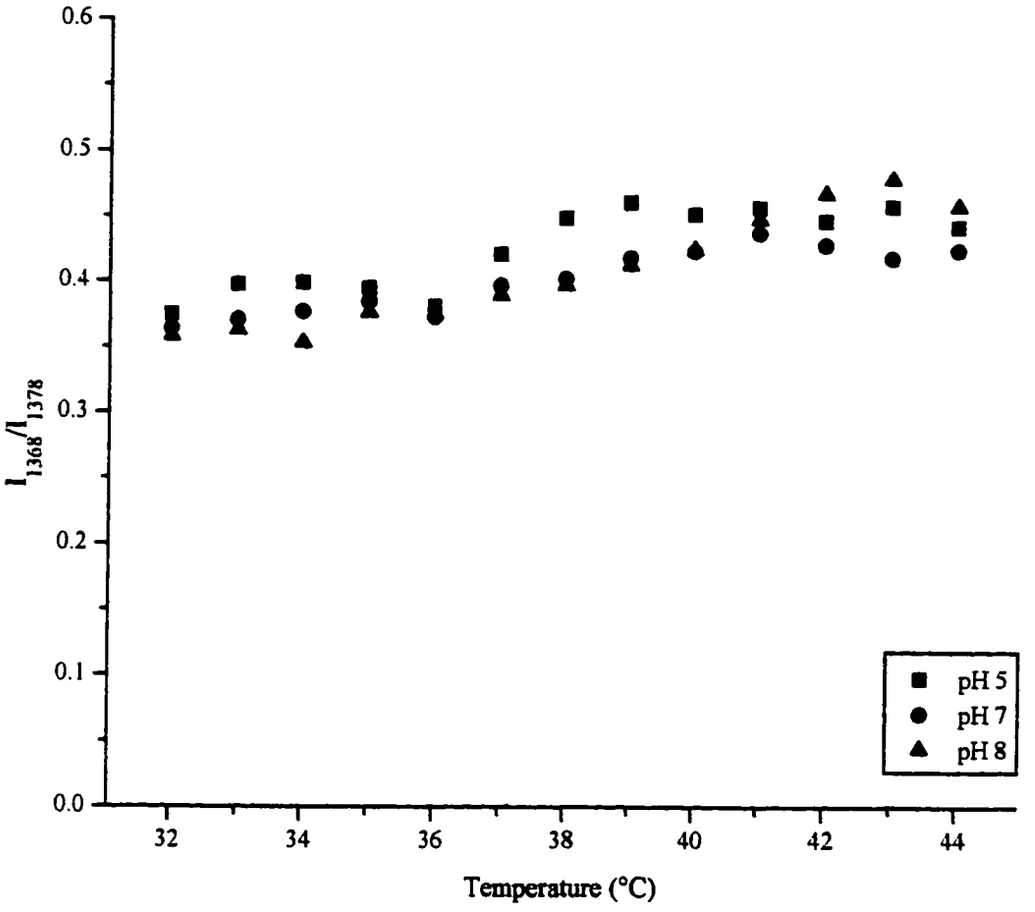

Figure 5.12: Phase Transition Profiles for the Kink Conformers of DPPC/CDC Samples Hydrated with 200 mM KH_2PO_4 Solutions at Various Initial pH's

hydrated with solutions having pH's of 5, 7, or 8. For the liquid crystalline phase, the CDC containing sample hydrated with a solution having an initial pH of 8 had the fewest number of gauche conformers when compared to similar samples hydrated with solutions having initial pH's of 5 and 7.

The phase transition profiles based on the number of end gauche conformers for pure DPPC hydrated with solutions having initial pH's of 5, 7 and 8 are given in Figure 5.13 for comparison. As can be seen, the phase transition profiles are very similar. The phase transition profiles indicate that there may be fewer end gauche conformers in the gel phase for pure DPPC hydrated with a solution having an initial pH of 5 than for pure DPPC hydrated with solutions having initial pH's of 7 or 8. In the liquid crystalline phase, there do not appear to be differences in the number of end gauche conformers for the different hydrating solutions. Similar results were obtained when monitoring the methylene symmetric stretching bands.

The phase transition profiles for the CDC containing samples hydrated with solutions having initial pH's of 5, 7, and 8 are given in Figure 5.14. The profiles suggest that CDC has less of an effect on the number of end gauche conformers in the liquid crystalline phase for the sample hydrated with a solution having an initial pH of 8 than on the samples hydrated with solutions having lower initial pH's. The percent differences show only minor (~3%) changes for the gel phase of the samples hydrated with solutions having initial pH's of 5, 7 and 8. Thus, the pH of the hydrating solutions does not appear to affect the number of end gauche conformers in the gel phase for CDC containing samples. These results are consistent with those obtained from monitoring the methylene

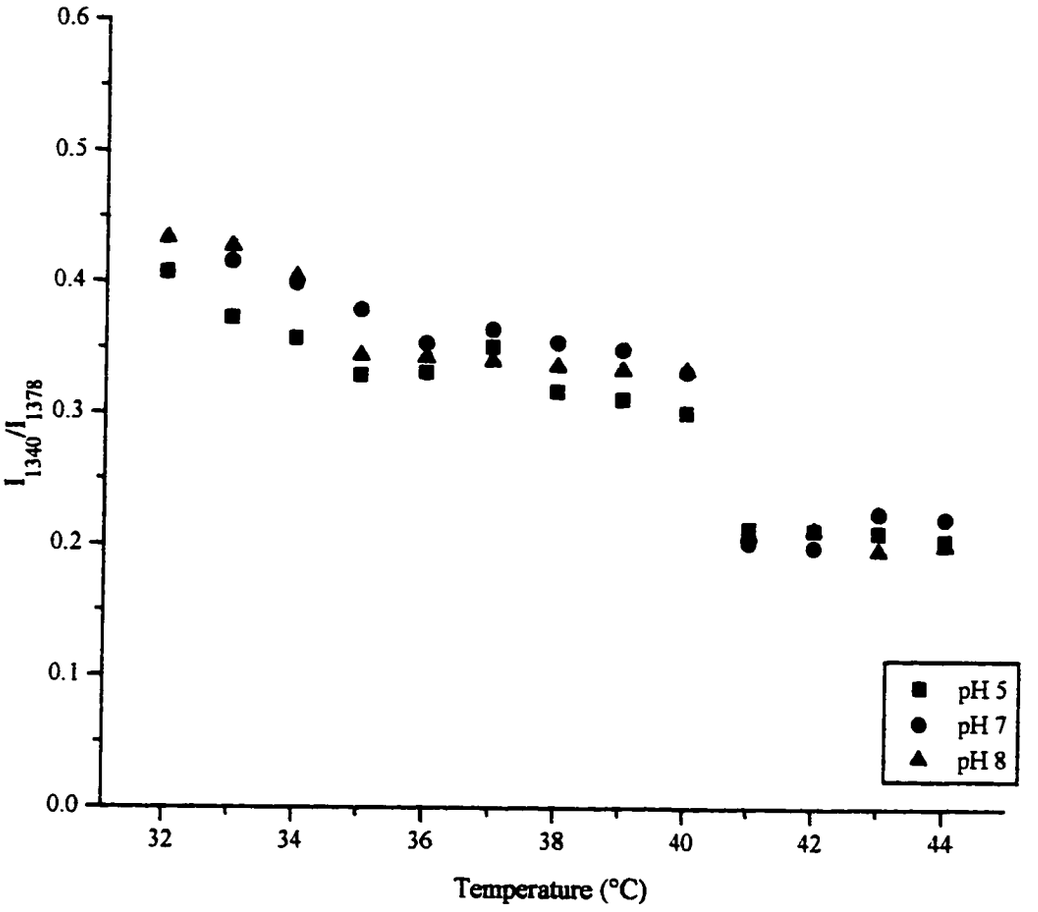

Figure 5.13: Phase Transition Profiles for the End Gauche Conformers of Pure DPPC Samples Hydrated with 200 mM KH_2PO_4 Solutions at Various Initial pH's

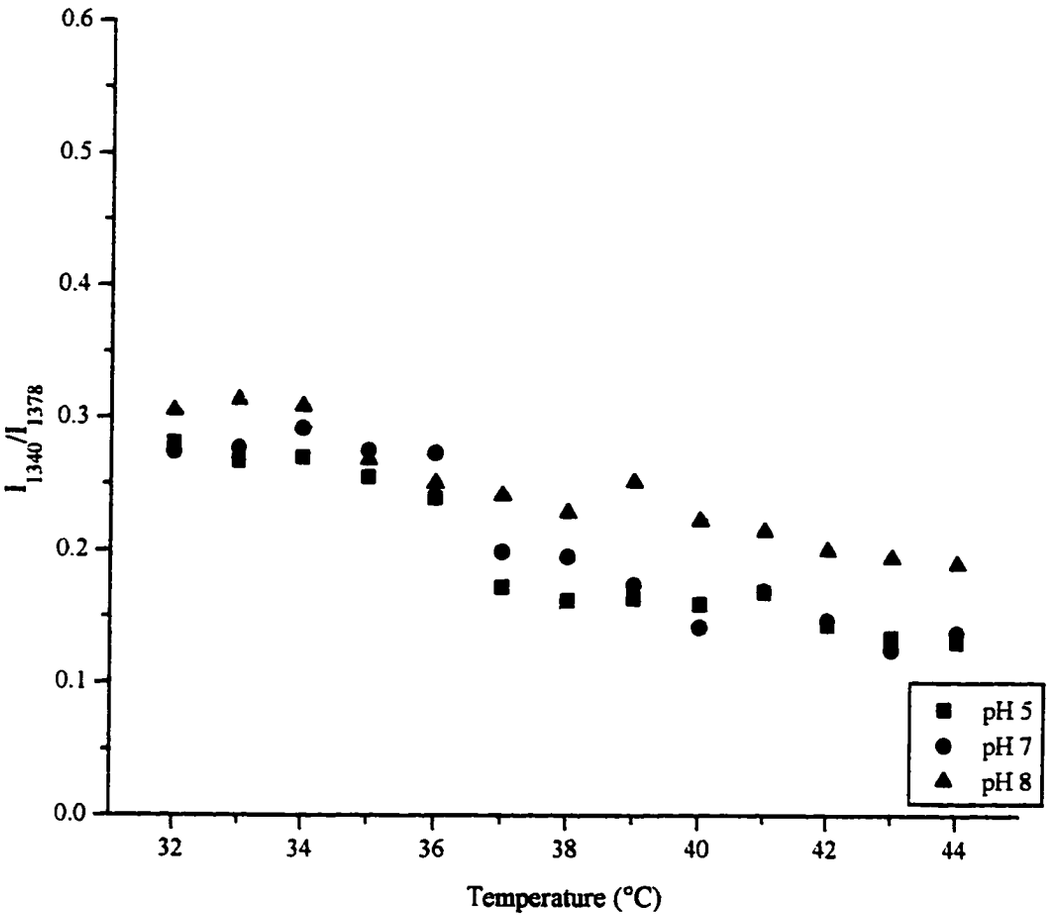

Figure 5.14: Phase Transition Profiles for the End Gauche Conformers of DPPC/CDC Samples Hydrated with 200 mM KH_2PO_4 Solutions at Various Initial pH's

symmetric stretching modes.

5.3.3 Effect of Adding Ethanol-d_6 to a 200 mM KH_2PO_4 Hydrating Solution Having an Initial pH of 8 on the Number and Type of Gauche Conformers Present in the Acyl Chains

Experiments were performed to determine the effect which the presence of ethanol-d_6 in the hydrating solution has on the number and type of gauche conformers present in pure DPPC and DPPC containing 15 mole percent CDC. The experiments were performed using 200 mM KH_2PO_4 hydrating solutions having an initial pH of 8 containing either 20 or 120 mg/ml ethanol-d_6. Plots of the I_{1368}/I_{1378} intensity ratio versus temperature for pure DPPC and DPPC samples containing 15 mole percent CDC hydrated with solutions (initial pH = 8) having ethanol-d_6 concentrations of 20 and 120 mg/ml are given in Figures 5.15 and 5.16, respectively. The I_{1368}/I_{1378} intensity ratio shows that the addition of 20 mg/ml ethanol-d_6 does not change the number of kink conformers, in the gel phase, for the pure DPPC sample when compared to pure DPPC hydrated with a solution containing no ethanol-d_6. There is a 0.003 (0.8%) difference in the I_{1368}/I_{1378} intensity ratios for the gel phase between the CDC containing sample hydrated with a solution containing 20 mg/ml ethanol-d_6 and the CDC containing sample hydrated with a solution which contains no ethanol-d_6. An increase in the intensity ratio is observed in the liquid crystalline phase for pure DPPC. There is a slight (-2.6%) decrease in the I_{1368}/I_{1378} intensity ratio in the liquid crystalline phase determined between the CDC containing sample and a similar sample hydrated with a solution that contains no ethanol-d_6. The cooperativity of the lipid molecules during the phase transition does not appear to be

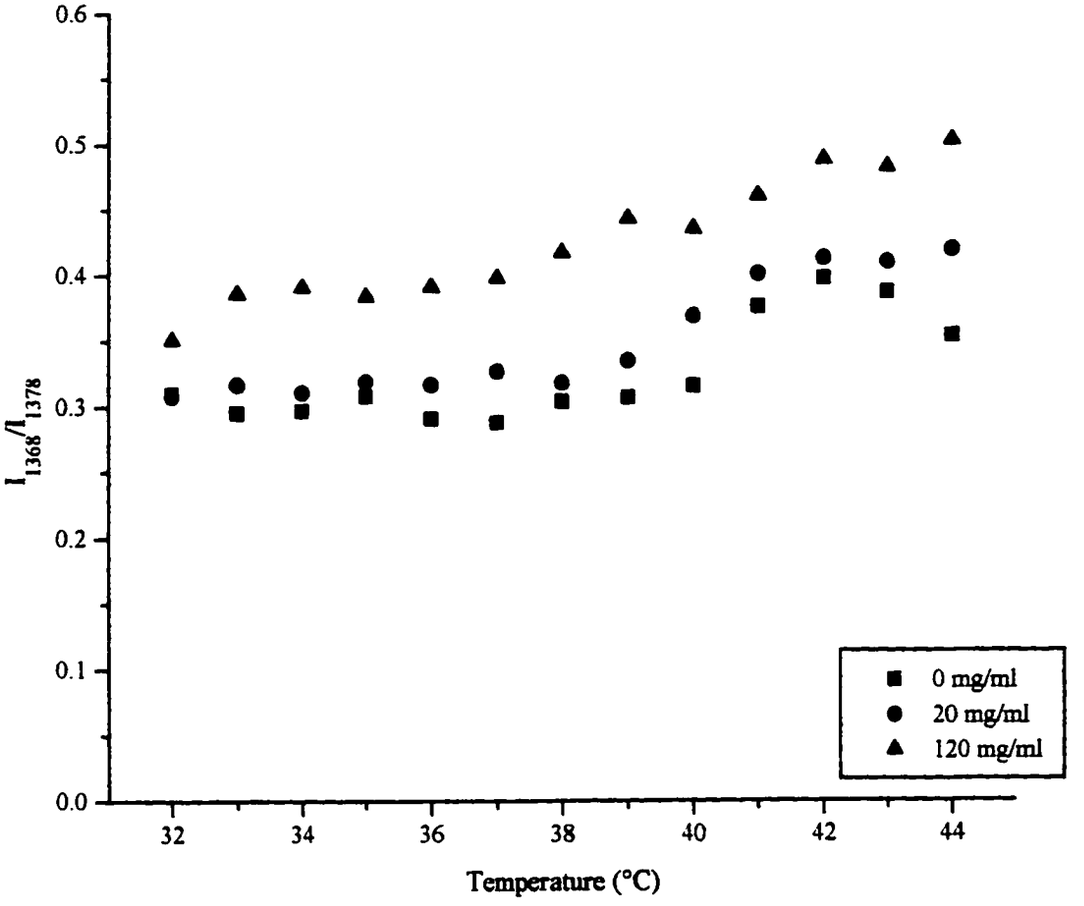

Figure 5.15: Phase Transition Profiles for the Kink Conformers of Pure DPPC Hydrated with Ethanol-d_6/200 mM KH_2PO_4 Solutions (Initial pH= 8)

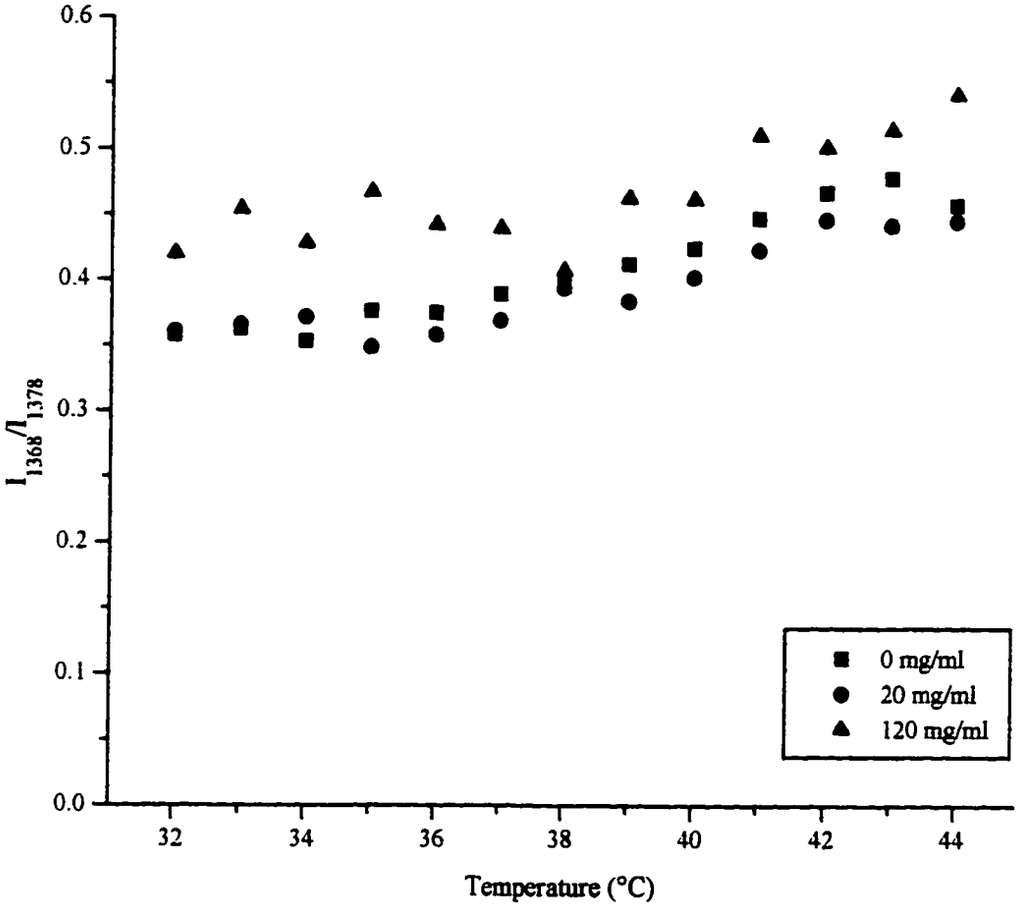

Figure 5.16: Phase Transition Profiles for the Kink Conformers of DPPC/CDC Samples Hydrated with Ethanol-d_6/200 mM KH_2PO_4 Solutions (Initial pH= 8)

affected by the presence of low concentrations of ethanol-d_6 in the hydrating solution. This result is different from the results based on the methylene symmetric stretching bands where a noticeable increase in the cooperativity of the lipid molecules during the phase transition was observed when even low concentration of ethanol-d_6 are added to the hydrating solution.

Plots of the I_{1340}/I_{1378} intensity ratio versus temperature for pure DPPC and DPPC samples containing 15 mole percent CDC hydrated with solutions (initial pH = 8) having ethanol-d_6 concentrations of 20 and 120 mg/ml are given in Figures 5.17 and 5.18. The I_{1340}/I_{1378} intensity ratio is lower in the gel phase for pure DPPC hydrated with a solution containing 20 mg/ml ethanol-d_6 compared to pure DPPC hydrated with a similar solution without ethanol-d_6. The I_{1340}/I_{1378} intensity ratio for the CDC containing sample hydrated with a solution containing 20 mg/ml ethanol-d_6 is similar to the I_{1340}/I_{1378} intensity ratio for the DPPC sample containing CDC hydrated with a solution containing no ethanol-d_6. The I_{1340}/I_{1378} intensity ratio remains approximately the same in the liquid crystalline phase for pure DPPC in comparison to pure DPPC hydrated with a solution containing no ethanol-d_6. Thus, the results of these experiments indicate that the presence of ethanol in the hydrating solutions increases the number of end gauche conformers in the gel phase but has little effect on the number of end gauche conformers in the liquid crystalline phase for pure DPPC. The I_{1340}/I_{1378} intensity ratio for the CDC containing sample in the liquid crystalline phase is similar to the I_{1340}/I_{1378} intensity ratio determined for the CDC containing sample hydrated with a solution which contains no ethanol-d_6. Thus, the presence of ethanol-d_6 in the hydrating solution for the CDC containing samples has

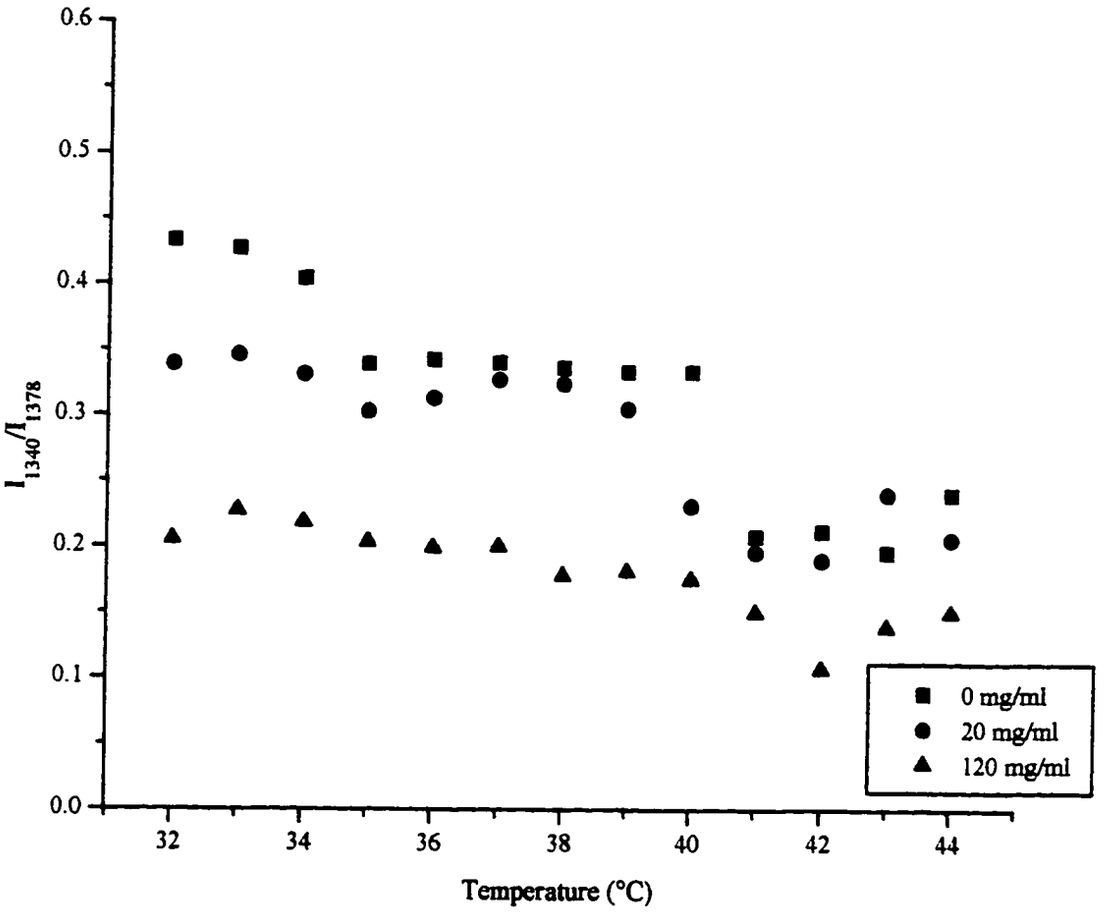

Figure 5.17: Phase Transition Profiles for the End Gauche Conformers of Pure DPPC Hydrated with Ethanol-d_6/200 mM KH_2PO_4 Solutions (Initial pH = 8)

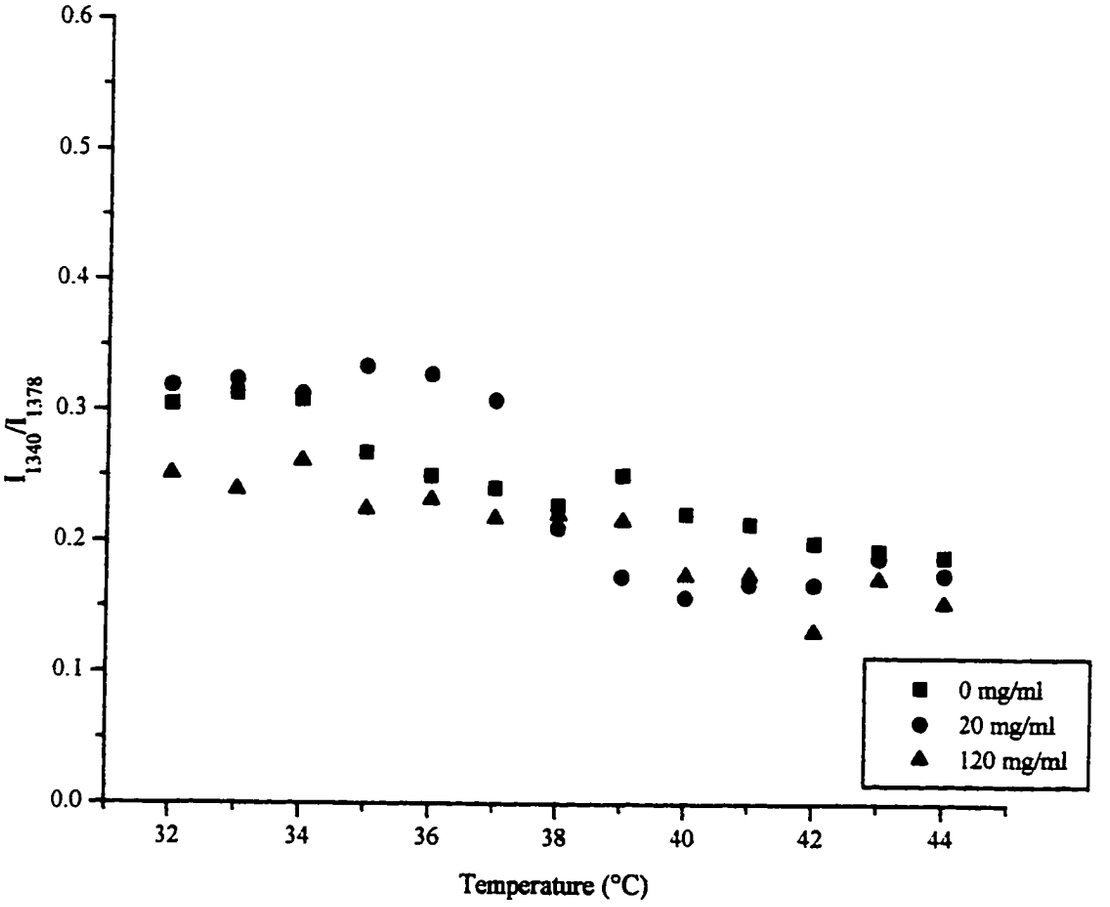

Figure 5.18: Phase Transition Profiles for the End Gauche Conformers of DPPC/CDC Samples Hydrated with Ethanol-d_6/200 mM KH_2PO_4 Solutions (Initial pH = 8)

relatively little affect on the number of end gauche conformers in both the gel and liquid crystalline phases.

The addition of 120 mg/ml ethanol-d_6 to the hydrating solution results in an interdigitated bilayer and an increase in the I_{1368}/I_{1378} intensity ratio for the gel and liquid crystalline phases of pure DPPC and for the DPPC sample containing CDC. The phase transition plots of the I_{1368}/I_{1378} intensity ratio versus temperature for pure DPPC and DPPC samples containing 15 mole percent CDC hydrated with solutions (initial pH = 8) having ethanol-d_6 concentrations of 20 and 120 mg/ml are given in Figures 5.15 and 5.16. The differences in the I_{1368}/I_{1378} intensity ratio for these samples and similar samples hydrated with a similar solution containing no ethanol-d_6 are large, indicating that the presence of ethanol-d_6 in the hydrating solution effects the number of kink conformers in the acyl chains. Thus, at high ethanol-d_6 concentrations, the number of kink conformers in the lipid's acyl chains increases in the both phases for pure DPPC and DPPC containing 15 mole percent CDC. The cooperativity of the lipid molecules during the phase transition does not appear to be affected by the presence of 120 mg/ml ethanol-d_6 in the hydrating solution. This result is different from the results obtained by monitoring the methylene symmetric stretching bands. The results for the methylene symmetric stretching bands showed a noticeable increase in the cooperativity of the lipid molecules during the phase transition when 120 mg/ml of ethanol-d_6 is present in the hydrating solution.

The I_{1340}/I_{1378} intensity ratio decreases, by 0.23, in the gel phase for pure DPPC and by 0.05 for the DPPC sample containing CDC hydrated with a solution containing 120 mg/ml ethanol-d_6 compared to pure DPPC and DPPC with 15 mole percent CDC hydrated

with a solution which does not contain ethanol-d_6, respectively. Thus, the presence of 120 mg/ml ethanol-d_6 in the hydrating solution decreases the number of end gauche conformers in the gel phase. The I_{1340}/I_{1378} intensity ratio also decreases by 0.05, in the liquid crystalline phase, for pure DPPC hydrated with a solution containing 120 mg/ml ethanol-d_6 when compared to pure DPPC hydrated with a solution which does not contain ethanol-d_6. The CDC containing sample hydrated with a solution containing 120 mg/ml ethanol-d_6 decreases by 0.04 in the liquid crystalline phase compared to a CDC containing sample hydrated with a solution containing 0 mg/ml ethanol-d_6. Thus, the results indicate that the addition of 120 mg/ml ethanol-d_6 decreases the number of end gauche conformers in the gel and liquid crystalline phases for both pure DPPC and the CDC containing sample.

5.3.4 Experiments Performed to Determine the Origin of the Band at 1329 cm^{-1} in Spectra of the Lipid Bilayers

Very interesting, and unexpected results were obtained by monitoring the band at 1329 cm^{-1}. The exact origin of this band is unknown at this time, though it is reasonable to expect that this band results from either a wagging mode or a methyl or methylene in the headgroup. Casal and McElhaney indicated a band in this region as being due to either the methylene or methyl in the headgroup of the lipid though they did not utilize this band because of its low intensity.[71] A band at ~1336 cm^{-1} was noted by O'Leary and Levin [111] and Silvius et al. [115] in Raman studies and was assigned to deformation modes of a methylene in the headgroup. An experiment was performed to determine if the 1329

cm^{-1} band is present in the spectrum of hexadecane. Hexadecane was chosen for these experiments since it is a long chain alkane and does not contain a headgroup. Spectra of neat hexadecane were acquired at 1 °C intervals over the temperature range from 32 to 44 °C. The absorbance spectrum obtained for hexadecane at 32 °C in the 1400-1320 cm^{-1} region is given in Figure 5.19. The contributions due to the individual absorption bands, as determined by the fitting program, are also presented in this figure. There is no evidence of a band at about 1329 cm^{-1} in the spectra of hexadecane at any of the temperatures studied. Thus, it is reasonable to conclude that the band at 1329 cm^{-1} in the spectrum of hydrated DPPC is either due to a CH_2 or CH_3 in the headgroup of the lipid as suggested by previous studies [71, 111, 115] or to a wagging mode, which is only observed in the more rigid gel phase of the lipid bilayer. Additional experiments using selectively deuterated lipids will need to be performed to determine the exact origin of this band.

5.3.5 Evaluation of the Effects which the Presence of Ethanol-d_6 in the Hydrating Solution and the Presence of CDC have on the I_{1329}/I_{1378} Intensity Ratio

Evaluation of the spectral data obtained for the pure DPPC shows that the I_{1329}/I_{1378} intensity ratio decreases with increasing temperature. At temperatures above the gel to liquid crystalline phase transition of the lipid bilayer the intensity of the band at 1329 cm^{-1} decreases to the point where it is no longer observable in the spectrum. This effect can best be observed by comparing the spectra given in Figures 5.2 and 5.20 for pure

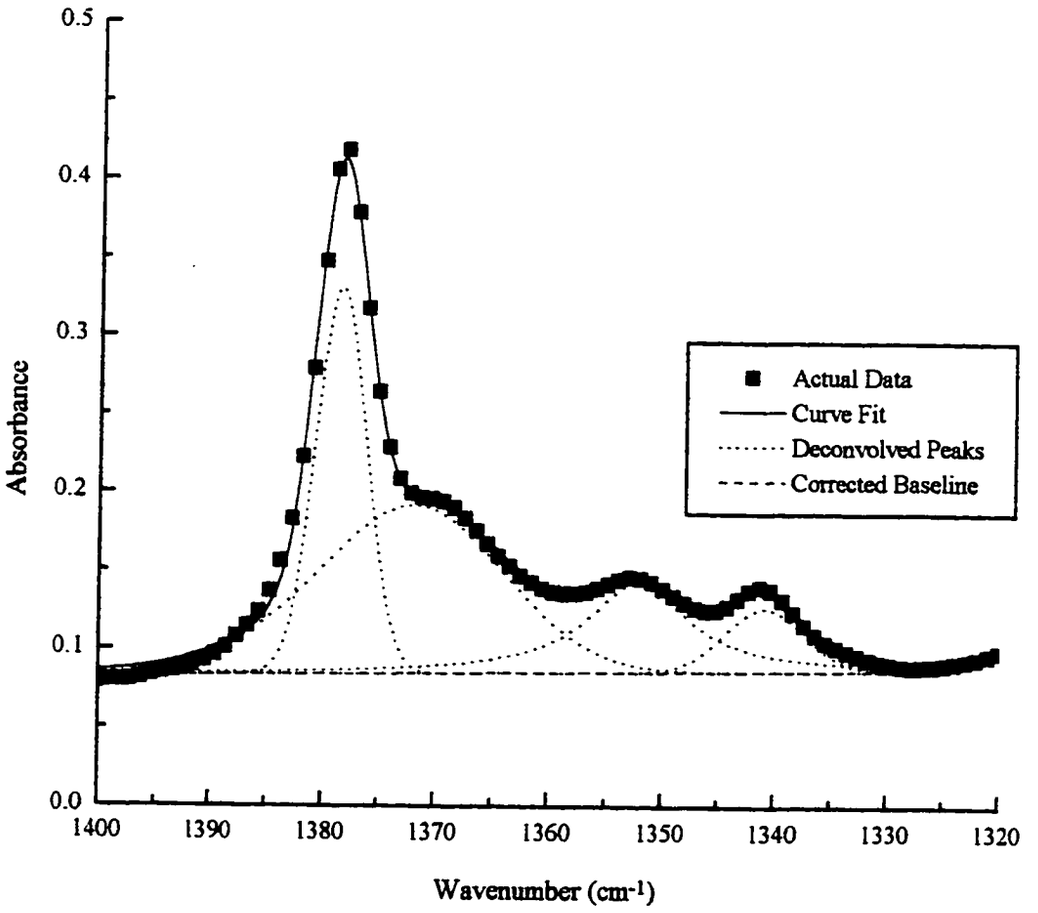

Figure 5.19: Absorbance Spectrum of Neat Hexadecane Acquired at a Temperature of 32 °C

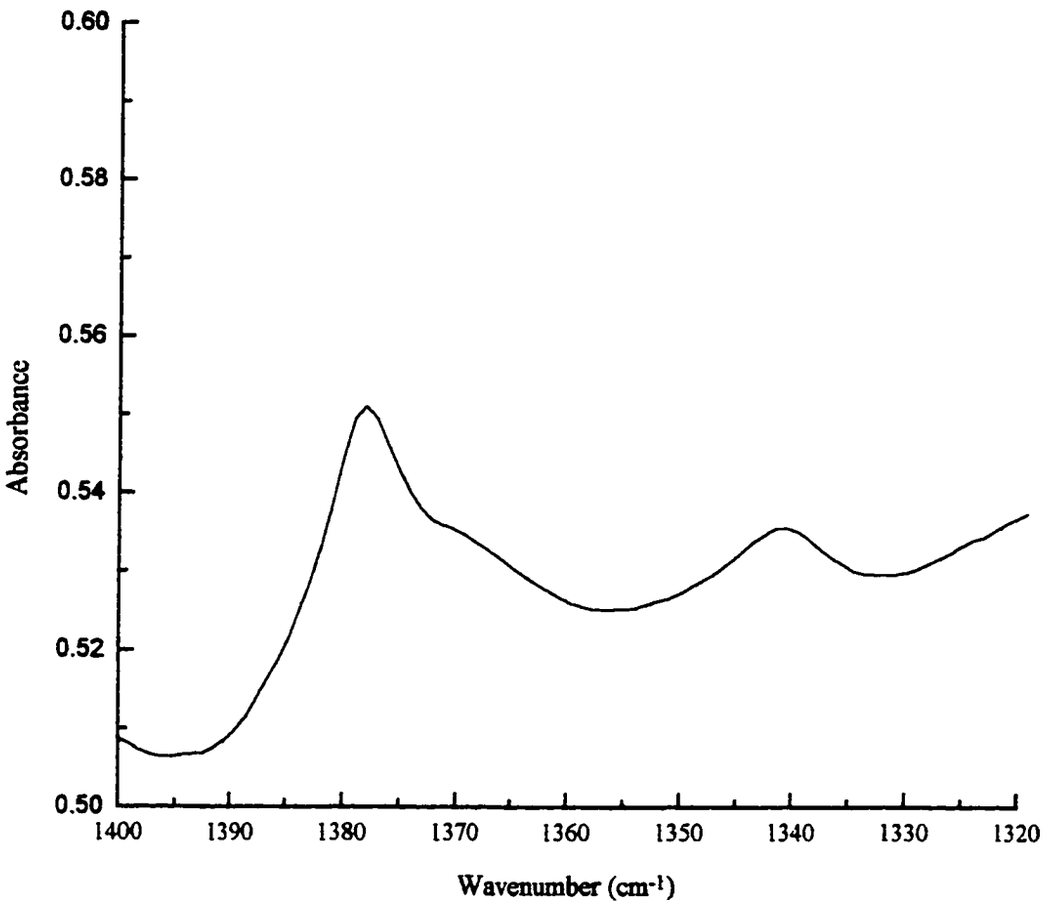

Figure 5.20: Absorbance Spectrum of Pure DPPC Hydrated with a 200 mM KH_2PO_4 Solution (Initial pH = 7, 44 °C)

DPPC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 7 acquired at temperatures of 32 and 44 °C. Since the band is not evident in the spectra acquired at higher temperatures, where the lipid is more fluid, these data are consistent with the band being due to a wagging mode. The I_{1329}/I_{1378} intensity ratio also decreases with the addition of CDC at temperatures below the phase transition temperature of the DPPC sample containing 15 mole percent CDC. The I_{1329}/I_{1378} intensity ratio appears to be lowest for the gel phase of samples containing CDC hydrated with a 200 mM KH_2PO_4 solution having an initial pH of 8. This trend can be observed in the plot of I_{1329}/I_{1378} intensity ratio versus temperature for DPPC samples containing 15 mole percent CDC hydrated with solutions having initial pH's of 5, 7, and 8 which is presented in Figure 5.21. The intensity ratio was found to decrease for samples hydrated with ethanol-d_6 containing solutions. At high ethanol-d_6 concentrations (120 mg/ml), no band is apparent at 1329 cm^{-1} in the spectrum of samples containing CDC for the gel phase as shown in the spectrum given in Figure 5.22. The I_{1329}/I_{1378} intensity ratios for pure DPPC and DPPC samples containing CDC hydrated under several conditions are given in Tables 5.3 and 5.4. Rowe [96] and Simon et al. [97] concluded that ethanol, at high concentrations interacts with the headgroup. Adding ethanol to the hydrating solutions, results in a decrease in the I_{1329}/I_{1378} intensity ratio. Previous results indicate that the bile salts are interacting with the headgroup, therefore, one would expect the I_{1329}/I_{1378} intensity ratio to decrease with the presence of bile salt in the lipid. Results have shown that the I_{1329}/I_{1378} intensity ratio decreases with the addition of the bile salt. Thus, the data are consistent with the interaction of the bile salts with the headgroup. Since the data collected through

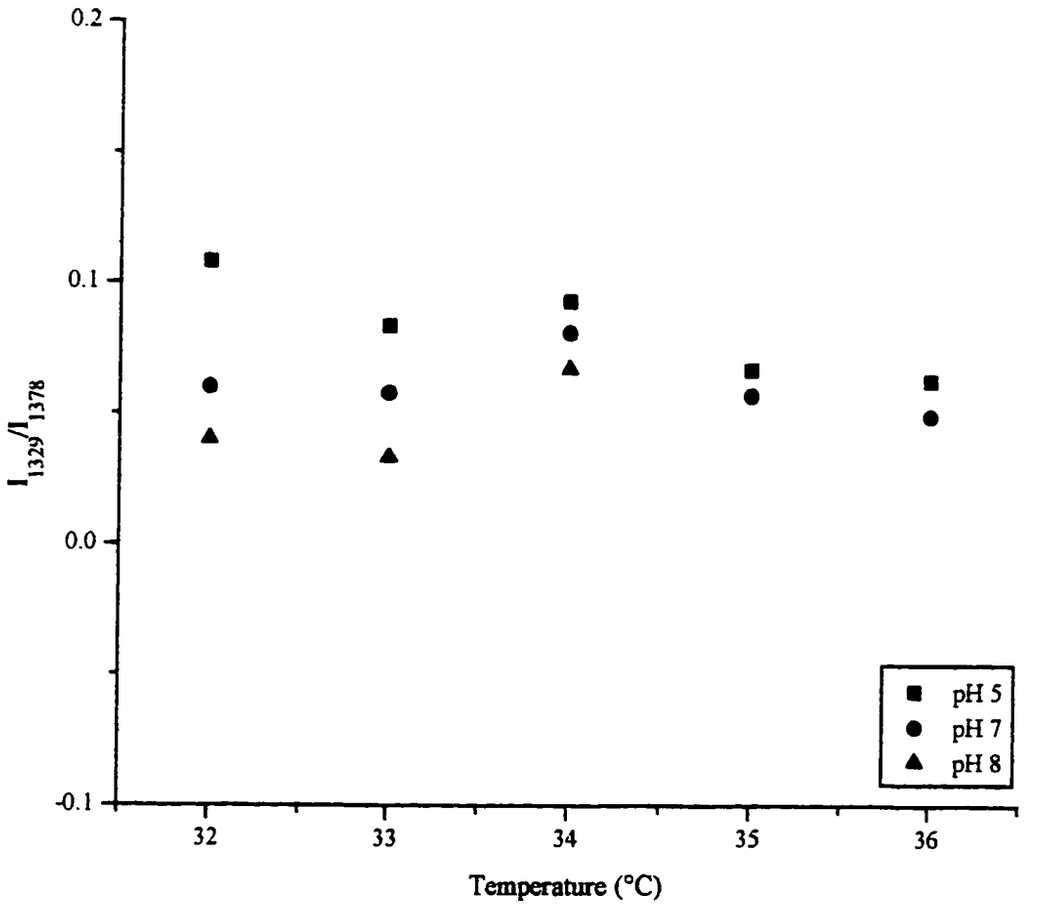

Figure 5.21: Plot of the I_{1329}/I_{1378} Intensity Ratios Versus Temperature for DPPC/CDC Samples Hydrated with 200 mM KH_2PO_4 Solutions at Various Initial pH's

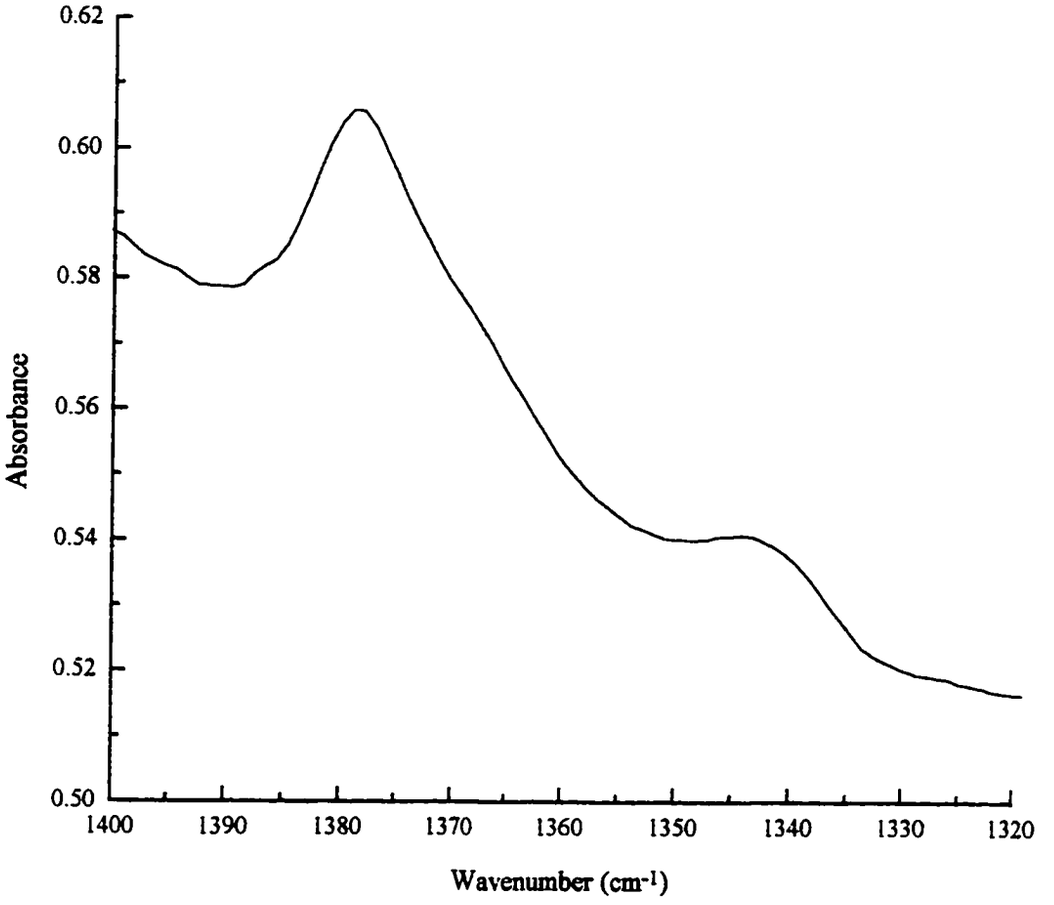

Figure 5.22: Absorbance Spectrum of a DPPC/CDC Sample Hydrated with a 120 mg/ml Ethanol-d_6/200 mM KH_2PO_4 Solution (Initial pH = 8, 32 °C)

Table 5.3: I_{1329}/I_{1378} Intensity Ratios for Pure DPPC and DPPC Samples Containing CDC Hydrated with 200 mM KH_2PO_4 Solutions (T = 32 °C)

Initial pH of Hydrating Solution	5	7	8
DPPC	0.145	0.154	0.154
CDC	0.108	0.060	0.040

Table 5.4: I_{1329}/I_{1378} Intensity Ratios for Pure DPPC and DPPC Samples Containing CDC Hydrated with Ethanol-d_6/200 mM KH_2PO_4 Solutions (Initial pH = 8, T = 32 °C)

Ethanol-d_6 (mg/ml)	0	20	120
DPPC	0.154	0.092	0.044
CDC	0.040	0.054	--------*

* No evidence of band in spectrum

these experiments and those for the hexadecane experiments show different results, the band at 1329 cm^{-1} needs to be further investigated.

5.4 Conclusion

Comparison of the results obtained using the methylene symmetric stretching modes and the methylene wagging modes provide significant information on the effects which the bile salts have on the DPPC bilayer. It should be noted that the methylene symmetric stretching data only provide information concerning the number of gauche conformers in the lipid's acyl chains not which particular type of gauche conformer is changing. The results obtained, for the DPPC bilayer in the liquid crystalline phase, for the kink conformers are similar to the results obtained for the DPPc bilayer in the liquid crystalline phase when the methylene symmetric stretching modes are utilized. That is, the number of kink conformers increases for the samples which contain CDC and the data obtained from the methylene symmetric stretching modes also indicates an increase in the number of gauche conformers. The number of end gauche conformers, however, decreases when CDC is present in the sample. The decrease in the number of end gauche conformers is not consistent with the methylene symmetric stretching mode data. It appears as if the two sets of data are in conflict. If, however, the bile salt molecules are interacting with the headgroup, then one can accept that there could be fewer end gauche conformers for the CDC containing sample than for pure DPPC. This is due to the fact

that end gauche conformers are located at the end of the acyl chains (ie., further into the

bilayer center) and CDC would have to distort significantly the acyl chain region in order to affect the end gauche conformers. Another difference between the two sets of data is that the methylene symmetric stretching modes showed little change in the number of gauche conformers in the gel phase while, the wagging modes suggest changes do occur in the gel phase of the DPPC bilayer.

The results provided by the two regions of the infrared spectrum for samples hydrated with the ethanol containing solutions also are similar. The information provided by the methylene symmetric stretching modes suggest an increase in the number of gauche conformers in the liquid crystalline phase for both pure DPPC and CDC containing samples. The number of kink conformers, at high ethanol concentrations, was also shown to increase in the liquid crystalline phase. However, the number of end gauche conformers was shown to decrease. Once again, the discrepancies between the two data sets may be due to the fact that the methylene symmetric stretching bands do not provide information regarding the type of gauche conformer that is changing. Further investigation of the end gauche conformers is necessary since the results presented are not consistent with the data in the literature.[70]

The results obtained indicate that the number of kink conformers increases and the number of end gauche conformers decreases for samples containing 15 mole percent CDC compared to pure DPPC samples hydrated under similar conditions. Since the end gauche conformers are located at the end of the acyl chains and the number is decreasing slightly when CDC is present in the samples, it suggests that CDC is not located in the acyl chain region. The results of experiments performed by Senak et al. [70] showed a decrease in

the number of end gauche conformers for DPPE in comparison to DPPC. They argued that the decrease in the number of end gauche conformers was due to the tighter packing of the lipid molecules in DPPE than in DPPC. A similar effect may occur for samples containing CDC if CDC is indeed interacting with the headgroup. CDC may cause the molecules to pack tighter if it is located between the headgroups of two lipid molecules. This tighter packing of the molecules may result in a decrease in the number of end gauche conformers in the lipid's acyl chains. The increase in the number of kink conformers may be due to the interaction between the headgroup and CDC. The acyl chains may have to kink in order to accommodate the disruption to the headgroup by CDC.

If the band at 1329 cm^{-1} is indeed due to a methyl or methylene in the headgroup, then the results obtained for this band may provide additional confirmation that CDC is indeed interacting with the headgroup of the lipid. The ratio of the intensity of the 1329 cm^{-1} band to the methyl umbrella mode was shown to decrease with the addition of CDC. The I_{1329}/I_{1378} intensity ratio was also shown to decrease more for the CDC containing sample hydrated with a solution having higher initial pH's (pH = 8). At high pH's, CDC exists primarily in the unprotonated form. This form of CDC would most likely prefer to interact with the more polar headgroup than the less polar, hydrophobic acyl chain region. The data obtained for ethanol containing hydrating solutions are also consistent with the interpretation that CDC is interacting with the headgroup. For samples containing CDC hydrated with solutions containing ethanol, the data show that the intensity ratio (I_{1329}/I_{1378}) decreases with increasing ethanol concentration suggesting that ethanol reduces the effects which the bile salts have on the lipid bilayer.

CHAPTER 6

CONCLUSION AND FUTURE STUDIES

6.1 Conclusion

The goal of this research was to investigate the effects which the unconjugated and conjugated forms of UDC and CDC have on the DPPC bilayer under various hydrating conditions. The results of this investigation provided additional information on the specific interactions which theses bile salts have with model membranes. Understanding the interactions of the bile salts with membranes is important due to the role of bile salts in human digestion and the use of selected bile salts as therapeutic agents.

The results of the studies presented here allow comparisons to be made among the effects which the three forms (unconjugated, taurine conjugated [116] and glycine conjugated) of the bile salt epimers, UDC and CDC, have on DPPC bilayers. In general, the results show that the addition of either of the bile salts, conjugated and unconjugated, to the DPPC bilayer results in a destabilization of the bilayer, a reduction in the cooperativity between the lipid molecules during the phase transition and an increase in the fluidity of the liquid crystalline phase. The results of the experiments also show that both the conjugated and the unconjugated forms of the CDC epimer destabilize the lipid assembly to a greater extent than the corresponding UDC epimer. The cooperativity between the lipid molecules during the phase transition for the unconjugated and the glycine conjugated UDC and CDC epimers is lower for samples hydrated with solutions having higher initial pH's than for samples hydrated with solutions having lower initial

pH's. A different effect was observed for the taurine conjugated UDC and CDC containing samples.[116] That is, no significant change in the cooperativity of the lipid molecules during the phase transition occurs with changes in the initial pH of the hydrating solution for the TCDC containing samples. The TUDC containing samples show a slight decrease in the cooperativity as the initial pH of the hydrating solution decreases. Based on these results, the interactions which the taurine conjugated bile salts have with the DPPC bilayer are affected less by the initial pH of the hydrating solution than either the glycine conjugated or the unconjugated bile salts. The result was not unexpected since the pH's of the hydrating solutions studied were all well above the pK_a of the taurine conjugated bile salts.

The effect which the presence of ethanol-d_6 in the hydrating solutions has on the bile salt/lipid interactions was also studied. Ethanol and the bile salts are present in the small intestine during the consumption of alcoholic substances. Ethanol, at high concentrations, is known to cause interdigitation of the lipid's acyl chains. The effect which the presence of ethanol-d_6 in the hydrating solutions has on the DPPC bilayer is similar for all bile salt containing samples. The data presented here, show that the presence of ethanol-d_6 in the hydrating solutions results in an increase in the cooperativity of the lipid molecules during the phase transition, and an increase in the fluidity of the liquid crystalline phase. At the highest ethanol-d_6 concentration studied, 120 mg/ml, the results of these experiments show that ethanol-d_6 subdues significantly the effects which the bile salts have on the DPPC bilayer. That is, for bile salt containing samples, ethanol at sufficiently high concentrations (ie., 120 mg/ml) was found to increase the stability of

the bilayer, increase the cooperativity of the lipid molecules during the phase transition and increase the level of acyl chain disorder (fluidity) in the liquid crystalline phase compared to bile salt containing samples hydrating with a solution containing no ethanol.

It was determined that the increase in the level of acyl chain disorder was due primarily to the increase in the number of gauche conformers.[1, 4, 13] Recently, Kodati et al. found that changes in the frequency of the methylene symmetric stretching bands may not be due solely to the introduction of gauche conformers into the lipid's acyl chains.[92] They indicate that other factors (such as interchain interactions) may result in a change in the frequency of these bands. The data collected for the wagging modes confirm that the number of gauche conformers, specifically the kink conformers, increases for samples containing CDC and for samples hydrated with solutions containing high concentrations of ethanol. Thus, it is reasonable to conclude that increases in the frequency of the methylene symmetric stretching modes for the lipid samples which contain bile salt are primarily due to the introduction of gauche conformers into the lipid's acyl chains.

The results of the studies presented here also provide evidence for the location of the bile salts in the lipid assembly. The data are consistent with the unconjugated bile salts being located at the aqueous interface region of the lipid.[42, 44] Thus, it appears that the bile salts interact with the headgroup. The data also indicate that the glycine and taurine conjugated bile salts interact with the headgroup. The interaction of the bile salts with the headgroup of the lipid also is consistent with the data obtained using ethanol containing hydrating solutions. Studies have indicated that ethanol, at high concentrations, interacts

with the headgroup.[96, 97] Since ethanol is present in higher concentrations than the bile salt, it is likely that the ethanol molecules favorably compete for the interaction sites of the headgroup. Thus, at the ethanol concentrations studied, the effect of ethanol on the bilayer predominates and the effect which the bile salts have on the bilayer is reduced.

The unconjugated form of UDC is used therapeutically for dissolving some types of gallstones. The results of these studies show that ethanol affects the interactions which UDC, in both the conjugated and unconjugated forms, has on the lipid bilayer. It is intriguing to note that ethanol and UDC can be present in the intestines of patients undergoing treatment with UDC. Based on these results, it reasonable to question what effect ethanol, if present in the intestines, has on the interactions between UDC and the cellular membranes of the intestines.

6.2 Future Studies

Many additional experiments could be performed to provide additional information as to the effect which bile salts have on lipid assemblies. For example, studies are needed to determine the effects which other bile salt species have on lipid assemblies. One bile salt which is particularly interesting is lithocholate. Lithocholate is a monohydroxy bile salt and is present in human bile at low concentrations. It is very hydrophobic and is known to be toxic. Clearly, the experimenter would have to exercise considerable caution when handling these samples. Since lithocholate is more hydrophobic than either of the two bile salts studied, it would be interesting to determine if lithocholate interacts with the bilayer in a manner similar to that of either UDC or CDC (ie., does lithocholate interact

with the headgroup or can it penetrate further into the bilayer). It would also be interesting to study the effects which the sulfated and amidated forms of lithocholate have on the lipid assembly since the sulfated form is not absorbed to a significant extent in the body.[18] These studies should be performed using hydrating solutions with several different initial pH's to determine if pH affects the interactions of these bile salts with the lipid bilayer since pH is an important variable in the gastrointestinal tract. Another bile salt which is of interest and should be studied is muricholate. Muricholate is a bile salt which is typically found in rats and has been suggested as having cytoprotective properties similar to that of ursodeoxycholate. Thus, it would be interesting to determine if muricholate interacts with the bilayer in a similar manner as UDC.

The experiments which employed the wagging modes to evaluate the interactions which the bile salts have with DPPC were performed only for CDC containing samples. These experiments should be extended to include studies of UDC and the taurine and glycine conjugated forms of both UDC and CDC. The conjugated forms of the bile salts are more polar than the unconjugated form. The conjugated bile salts are known to interact with the headgroup.[44] If the conjugated bile salts interact with the headgroup in a different manner than the unconjugated bile salts, then it is possible that conjugated bile salts might affect the number of end gauche conformers and possibly even the number of kink conformers into the lipid's acyl chains. If it were found that fewer kink conformers were formed, this might provide evidence that the bile salt is lying on top of the headgroups and not actually inserting between two headgroups. It would also be interesting to note how the conjugated bile salts affect the intensity of the band at 1329

cm^{-1} since this band is believed to be due to either CH_2 or CH_3 in the headgroup. It is expected that the taurine and glycine conjugated forms of UDC and CDC will cause a decrease in the intensity of this band due to their increased polarity. It is postulated that the decrease will be greater than the decrease observed with unconjugated CDC since the conjugated bile salts will have a greater effect on the headgroup.

Finally, all of the experiments described here were performed using multilamellar vesicles (MLVs) composed of DPPC. Studies should be performed using small unilamellar vesicles (SUVs) since they more closely model real membrane systems. Once these experiments are completed and the effects of the bile salts on model membranes are well understood, studies should be extended to determine the effects of selected bile salts species on real membranes.

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APPENDIX

APPENDIX A

LIST OF DATA FILES

	Sample	Filename
50 mM	DPPC/0/7	lib32
	DPPC/20/7	liet78
	DPPC/55/7	eth86
	DPPC/120/7	lib31
	DPPC/0/5	lec35
	DPPC/20/5	lec44
	DPPC/55/5	lec59
	DPPC/120/5	lec54
	DPPC/0/8	leb80
	DPPC/20/8	leb93
	DPPC/55/8	lec08
	DPPC/120/8	leb96
	UDC/0/7	lib43
	UDC/20/7	liet76
	UDC/55/7	eth82
	UDC/120/7	lib24
	UDC/0/5	lec36
	UDC/20/5	lec45
	UDC/55/5	lec60
	UDC/120/5	lec56
	UDC/0/8	leb76
	UDC/20/8	lec15
	UDC/55/8	lec09
	UDC/120/8	leb97
	CDC/0/7	lib44
	CDC/20/7	liet95
	CDC/55/7	liet89
	CDC/120/7	lib27

	Sample	Filename
	CDC/0/5	lec37
	CDC/20/5	lec46
	CDC/55/5	lec61
	CDC/120/5	lec57
	CDC/0/8	leb86
	CDC/20/8	lec42
	CDC/55/8	lec10
	CDC/120/8	leb98
200 mM	DPPC/0/7	lec97
	DPPC/20/7	led37
	DPPC/120/7	led28
	DPPC/0/5	lec99
	DPPC/20/5	led44
	DPPC/120/5	led47
	DPPC/0/8	led05
	DPPC/20/8	led33
	DPPC/120/8	led23
	UDC/0/7	lec89
	UDC/20/7	led35
	UDC/120/7	leg51
	UDC/0/5	lec93
	UDC/20/5	led50b
	UDC/120/5	led48
	UDC/0/8	led06
	UDC/20/8	led32
	UDC/120/8	led24
	CDC/0/7	lec88
	CDC/20/7	led36
	CDC/120/7	led30
	CDC/0/5	lec94
	CDC/20/5	led46
	CDC/120/5	led49

	Sample	Filename
	CDC/0/8	led07
	CDC/20/8	led31
	CDC/120/8	led25
GLYCO	DPPC/0/7	leg14
	DPPC/20/7	leg08
	DPPC/120/7	lef61
	DPPC/0/5	lef10
	DPPC/20/5	leg10
	DPPC/120/5	lef47
	DPPC/0/8	lef10b
	DPPC/20/8	lef76
	DPPC/120/8	leg20
	GUDC/0/7	leg27
	GUDC/20/7	lef56
	GUDC/120/7	lef62
	GUDC/0/5	leg38b
	GUDC/20/5	leg39b
	GUDC/120/5	lef71
	GUDC/0/8	lef87b
	GUDC/20/8	leg13b
	GUDC/120/8	leg22b
	GCDC/0/7	leg28b
	GCDC/20/7	leg09
	GCDC/120/7	lef63
	GCDC/0/5	leg15
	GCDC/20/5	lef68b
	GCDC/120/5	lef72
	GCDC/0/8	lef12
	GCDC/20/8	lef78
	GCDC/120/8	leg22

	Sample	Filename
Spectral Contribution	DPPC	lef25
	w/KH_2PO_4	lef25b
	w/2.5 mM CDC	lef26b
	w/5.0 mM CDC	lef27b
	w/10.0 mM CDC	lef28b
	w/20.0 mM CDC	lef29b
	w/50.0 mM CDC	lef30b
	w/100.0 mM CDC	lef31b
	KH_2PO_4	lef26
	2.5 mM CDC	lef27
	5.0 mM CDC	lef28
	10.0 mM CDC	lef29
	20.0 mM CDC	lef30
	50.0 mM CDC	lef31
	100.0 mM CDC	lef32
	DPPC	lef50
	w/KH_2PO_4	lef50b
	w/24 mg/ml ETOH	lef51b
	w/12 mg/ml ETOH	lef52b
	w/4 mg/ml ETOH	lef53b
	KH_2PO_4	lef51
	24 mg/ml ETOH	lef52
	12 mg/ml ETOH	lef53
	4 mg/ml ETOH	lef54

	Sample	Filename
WAG	DPPC/0/7	leg57b
	DPPC/0/5	leg56
	DPPC/0/8	leg59
	CDC/0/7	leg58
	CDC/0/5	leg57
	CDC/0/8	leg60
	DPPC/20/8	leg61
	DPPC/120/8	leg63
	CDC/20/8	leg62
	CDC/120/8	leg64

Spectral Contribution to WAG

DPPC w/ KH_2PO_4	leg84b
	leg92b
DPPC w/ 4.6 mM CDC	leg85b
DPPC w/ 12.1mM CDC	leg86
DPPC w/ 29.0 mM CDC	leg87b